

Studier över Insulinsekretion, Insulinbindning till Erythrocyter och Insulinkänslighet med speciell hänsyn till diabetes mellitus typ I i tidigt skede hos barn

Sten A. Ivarsson



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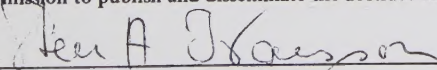
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Title and subtitle Studies on insulin secretion, insulin binding to erythrocytes and insulin sensitivity with special reference to type I diabetes mellitus of recent onset in children		
Abstract The purpose was to study various mechanisms which may explain the impaired insulin effect, which characterize the initial phase of type I diabetes mellitus. In the children with diabetes the C-peptide excretion was higher while the plasma C-peptide level was substantially lower as compared with normal controls. These findings indicate a higher urinary leakage of C-peptide at this phase of the disease. Follow-up studies indicated that the higher the level of the urinary C-peptide at admission, the longer the remission period and the less the insulin requirement after one year. The corrected C-peptide excretion correlated with the age of the patients indicating a more severe beta-cell damage in the younger patients. The influence of the aging of the erythrocyte on the insulin binding was studied. It was found that reticulocytes have higher binding capacity for insulin than mature erythrocytes. In patients with normal erythropoiesis the age-related factors constituted 35% and the methodological factors 5% of the variation, while the remaining 60% were independent of these factors. In patients with haemotopoetic disease with active erythropoiesis the insulin binding to erythrocytes and their pyruvate kinase activity had nearly the same half-life while the fall in the reticulocyte count was much more rapid. In the children with diabetes mellitus type I as well as in a child with superimposed Addison's disease the insulin binding to erythrocytes was decreased but was restored to normal in the remission phase and after treatment. The insulin sensitivity was studied using an insulin infusion test. It was demonstrated that there was a correlation between the blood glucose decrement and the severity of the diabetic state. When entering remission phase the retested children had a normal sensitivity. The findings indicate that insulin resistance is not an abnormality associated with diabetes mellitus per se.		
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STUDIES ON INSULIN SECRETION, INSULIN BINDING TO
ERYTHROCYTES AND INSULIN SENSITIVITY WITH SPECIAL REFERENCE TO
TYPE I DIABETES MELLITUS OF RECENT ONSET IN CHILDREN

AKADEMISK AVHANDLING

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Sten A. Ivarsson
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STUDIER ÖVER INSULINSEKRETION, INSULINBINDNING TILL
ERYTHROCYTER OCH INSULINKÄNSLIGHET MED SPECIELL HÄNSYN TILL
DIABETES MELLITUS TYP I I TIDIGT SKEDE HOS BARN

Sten A. Ivarsson

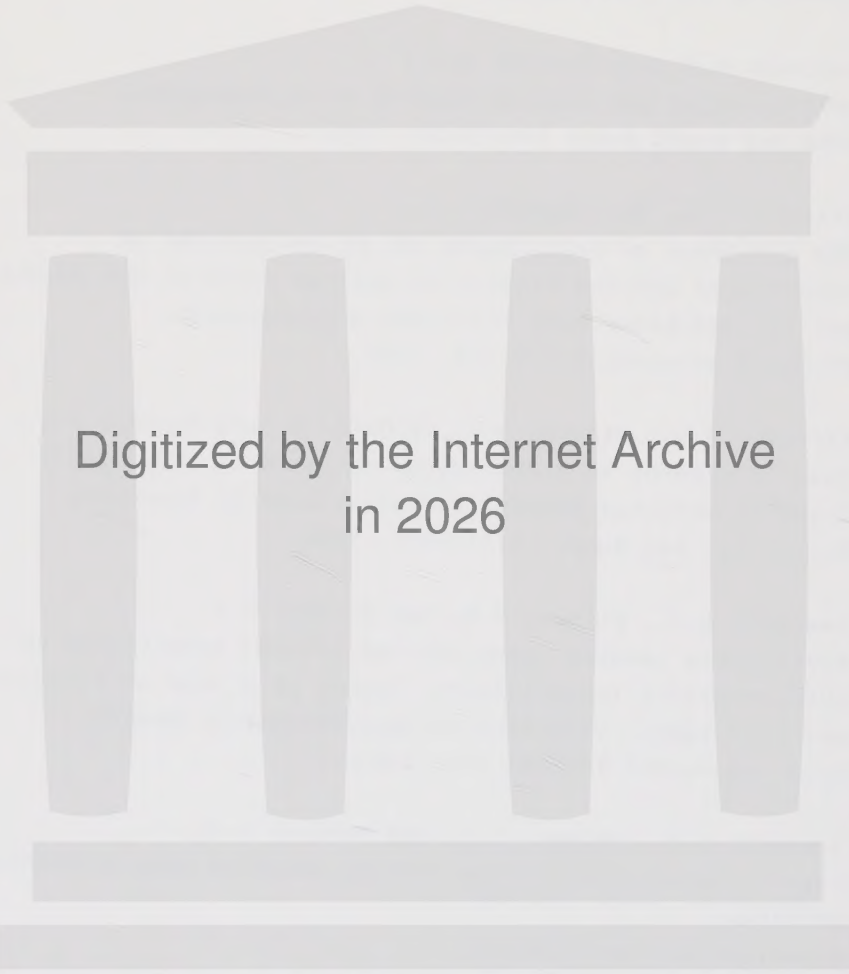
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- III. Ivarsson S.A. and Thorell J.I.:
The influence of erythrocyte age on estimations of erythrocyte insulin binding in healthy children and adults and in conditions with increased erythropoiesis.
Diabetes Research 2:249-253, 1985.
- IV. Ivarsson S.A., Nilsson K.O., Sjöblad S. and Thorell J.I.:
Insulin binding to erythrocytes in children with type I diabetes mellitus before, and after insulin treatment.
Horm Metab Res Suppl 16:171-174, 1984.
- V. Ivarsson S.A., Nilsson K.O. and Thorell J.I.:
Erythrocyte insulin receptors and insulin sensitivity in adrenocortical insufficiency. Report of a case of diabetes mellitus type I with superimposed Addison's disease.
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I texten kommer dessa delarbeten att bli refererade till genom användning av ovanstående numrering.



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Introduktion och bakgrund

Diabetes mellitus är ett syndrom där grunddraget är hyperglykemi. Olika undergrupper med olika patofysiologi och kliniska manifestationer existerar (Creutzfeldt et al 1976), och en enhetlig terminologi och klassifikation finns nu utarbetad (National Diabetes Data Group, 1979). I detta arbete kommer endast typ I diabetes mellitus hos barn att behandlas. Denna typ kännetecknas av en ofta abrupt insättande sjukdomsbild med ketosbenägenhet, och patienten är beroende av insulin för att överleva. Ett särdrag är dess remissionsbenägenhet efter det att insulinterapi har inletts, vilket innebär att insulinbehovet minskar eller t.o.m. tillfälligt kan upphöra. Denna remission kan vara från veckor till år. Patienterna har alltså i insjukningsskedet en absolut eller relativ insulinbrist, eftersom exogen tillförsel av insulin är nödvändig.

Insulin produceras i pankreas betaceller som pre-proinsulin. Denna peptid konverteras snabbt till proinsulin, som i sin tur i betacellens granula klyvs till insulin och C-peptid (Steiner & Oyer 1967, Steiner et al 1969). Insulin och C-peptid insöndras till cirkulationen i ekvimolära mängder (Rubenstein et al 1969). Insulin är en intercellulär budbärare som förmedlar en signal från pankreasöarnas betaceller till målorganen i kroppen. Då insulin når dessa, verkar det genom flera steg (Blecher & Bar 1981):

- bindning av hormonet till dess specifika receptorer på ytan av cellens plasmamembran,
- aktivering av receptorn genom interaktion mellan hormonet och receptorn, vilket initierar en signal genom membranen,
- generering av det intracellulära budskapet,
- förändringar i transportsystemet och enzymerarnas aktivitet som resulterar i en metabolisk effekt.

Vår kunskap om insulinets verkan på molekylär nivå är ofullständig, då vår möjlighet att mäta dessa biokemiska steg och deras inbördes relationer ännu är begränsad.

I insjukningsfasen kan sjukdomen sålunda vara ett uttryck för:

- en sekretionsrubbing dvs. insulinbrist,
- en nedsatt förmåga att binda insulin till dess receptor,
- en förändring av cellens insulinkänslighet på postreceptornivå.

I början av detta århundrade trodde man att insulinkrävande diabetes mellitus var uttryck för en total betacellsdestruktion. Senare kliniska observationer, framför allt hos barn, visade emellertid att dessa ofta fick en remissionsperiod, och somliga barn kunde t.o.m. undvara insulin under flera månader, vilket talade för en funktionell återhämtning av betacellsfunktionen (Brush 1944). Genom utveckling av radioimmunologiska metoder blev det möjligt att mäta insulin i plasma (Yalow & Berson 1960) och därigenom bedöma insulinsekretionen. Vid studier av nyinsjuknade barn med diabetes mellitus fann man ingen stegring av plasma-insulinnivåerna vid olika provokationstest (Baker et al 1967, Parker et al 1968), vilket talade för en upphävd insulinsekretion. Ej heller kunde man påvisa stegring av insulinnivåer i den efterföljande remissionsperioden vid provokationstest (Baker et al 1967, Illig och Prader 1968). I andra studier hävdades dock att remissionsperioden innebar en viss återhämtning av betacellernas insulinproduktion (Johansen & Ørskov 1969, Weber 1972). Cirku- lerande insulinantikroppar inducerade av insulinbehandling gjorde det dock problematiskt att använda insulinmätningar för bedömning av betacellsfunktionen. I och med att bestämning av C-peptid i plasma blev möjlig, erbjöds nya möjligheter att påvisa en eventu-uell kvarstående insulinproduktion.

Genom studier av C-peptidnivåerna som mått på kvarvarande beta-cellfunktion, kunde Block och medarbetare visa (1972, 1973) att den låga eller uteblivna endogena insulinproduktionen vid insjuknandet till en del reflekterar en funktionell oförmåga och inte en total brist på insulin. De kunde sålunda påvisa mätbara C-peptidnivåer vid förnyad stimulationstest i remissionsperioden. Numera torde det vara belagt, att övervägande delen av patienterna har en kvarvarande om än ringa insulinproduktion vid sjukdomsdebuten av typ I diabetes mellitus (Ludvigsson & Heding 1978, Madsbad et al 1980, Menchini et al 1980).

I början av 1970-talet påvisades för första gången med hjälp av ^{125}I -insulin att insulin kunde bindas till receptorer i plasmamembraner hos råttlever (Freychet et al 1971) och på isolerade fettceller hos råttor (Cuatrecasas 1971). Fortsatta studier visade att denna insulinbindning medförde metabola effekter (Kono & Barham 1971): Alltsedan dess har studiet av insulinbindningen till ytcellsreceptorer varit intensiv. Tillgängliga experimentella data talar för att insulinreceptorn har två huvudfunktioner:

- dels att igenkänna hormonet och binda detta till sig,
- dels att genom interaktion med hormonet åstadkomma en aktivering av cellulära processer såsom transport och metabolism av glukos (Blecher & Bar 1981).

Den mest betydelsefulla faktorn, som reglerar insulinreceptor-koncentrationen, är hormonet själv. Sålunda har man experimentellt visat att odlade lymfocyter (Gavin III et al 1974), hepatocyter (Blackard et al 1978), fibroblaster (Mott et al 1979) och adipocyter (Livingston et al 1978, Marshall & Olefsky 1980) får en tidsberoende reduktion av bindningen till insulinreceptorerna vid höga insulinkoncentrationer, s.k. "nerreglering", medan låga insulinnivåer medför en ökad insulinbindning, s.k. "uppreglering" (Le Marchand-Brustel & Freychet 1979, Davidson & Kaplan 1977, Kobayashi & Olefsky 1979, Hepp et al 1975, Kasuga et al 1978).

Hos människan beskrevs insulinreceptorer först i studier på odlade fibroblaster och isolerade blodceller (Gavin III et al 1972). Givetvis vore det önskvärt att kunna studera insulinreceptorer vid diabetes mellitus på de viktigaste målorganen för insulin såsom lever-, muskel- och fettceller. Detta stöter dock på praktiska svårigheter och etiska problem. Man har därför studerat insulinreceptorn huvudsakligen på andra mer lättillgängliga celltyper såsom monocyter, erytrocyter och fibroblaster i förhoppningen att deras receptorstatus avspeglar de klassiska målorganens tillstånd.

Redan 1932 påpekade Himsworth att insulin tycks variera i sin verkan hos olika patienter med diabetes mellitus (Himsworth 1932), och fyra år senare föreslog han att man borde indela diabetes-

patienterna i insulinkänsliga och insulinokänsliga på bas av de blodsockersvar man fick då insulin administrerades omedelbart efter en oral glukosbelastning (Himsworth 1936).

Studier av förändringar i insulinkänslighet vid olika metabola tillstånd kräver möjlighet att mäta plasmainsulinkoncentrationen och samtidigt insulinberoende metabola processer. Utvecklingen av radioimmunoassay (Yalow & Berson 1960) möjliggjorde en känslig specifik mätning av plasmainsulin, och därefter har olika metoder för att bestämma in vivo-effekten av förändringar i plasma-insulinnivån introducerats.

Avsikten med denna studie var att undersöka olika mekanismer, som kan ligga bakom den bristande insulinverkan som föreligger i samband med insjuknandet i diabetes mellitus typ I hos barn. Som ett mått på kvarvarande insulinproduktion studerades C-peptid-utsöndringen i urin. Erytrocytmodellens tillämpbarhet för studier av insulinbindning undersöktes ur aspekten hur erytrocytens åldrande påverkar insulinbindningen. Insulinbindning till erytrocyter som ett mått på insulinreceptormängden studerades hos nyinsjuknade barn med diabetes mellitus före insatt behandling och i remissionsfas. Insulinkänsligheten undersöktes i insjukningsfasen och relaterades till parametrar som avspeglar det diabetiska tillståndet.

MATERIAL OCH METODIK

Sammanlagt ingick 55 nyinsjuknade barn med typ I diabetes mellitus i studien, varav 19 i C-peptidstudien (delarbete I), 24 i insulinreceptorstudien (delarbete IV) samt 21 i insulinkänslighetsstudien (delarbete VI). En patient studerades dessutom 4 år efter sin diabetesdebut i samband med en begynnande binjurebarksinsufficiens (delarbete V). I patientmaterialet ingick dessutom 4 vuxna med ökad erytropoes (delarbete III).

I delarbete II ingick 2 friska barn och 2 nyfödda med hyperviscositetssyndrom, 3 friska vuxna och 4 vuxna med olika sjukdomar såsom anemi, hjärtfel och levercirros.

Normalpersonerna utgjordes av 19 barn i C-peptidstudien (delarbete I), 10 barn samt 14 vuxna i receptorstudierna (delarbete III och IV) samt 20 barn insulinkänslighetsstudien (delarbete VI).

METODIK

C-peptid i urin

En dygnsmängd urin samlades i flaskor som förbehandlats med en lösning bestående av 100 g bovint serumalbumin, 0,1 g natriumazid och 0,2 g Merthiolat[®] per liter i 0,05 M fosfatbuffert, pH 7,5. Dessa flaskor förvarades vid 5°C under samlingsdygnet. Efter insamlandet överfördes de omedelbart till laboratoriet, där mängden uppmättes och 10 ml överfördes till provrör och förvarades vid -20°C under högst 3 veckor, innan analysen skedde. Urinen späddes med 6% humant serum albumin i 0,05 M fosfatbuffert, pH 7,5, och C-peptidkoncentrationen bestämdes i tre spädningar, 1:20, 1:50 och 1:100. Analysen utfördes enligt Heding (1975) med antiserum M1230 (Faber et al 1976). Värdena uttrycktes i nmol/dygn/m² kroppsytta. Detektionsgränsen var 0,05 nmol/L. Metodfelet för C-peptidanalysen var 4,3% inom dubbelbestämningar och 9,6% mellan olika bestämningstillfällen.

C-peptid i plasma

10 ml venblod i EDTA-rör centrifugerades och plasma förvarades vid -20°C. Analysen utfördes enligt Heding (1975) med antiserum M1230 (Faber et al 1976) inom tre veckor efter provtagningen.

Detektionsgränsen var 0,05 nmol/L.

Receptorstudier på erythrocyter

Erythrocytpreparationen

10 ml blod samlades i heparinrör och analyserades inom 24 timmar. Efter centrifugering av blodet (10 minuter, 400xg, 20°C) borttogs plasman och de kvarvarande röda blodkropparna blandades med lika del fysiologisk NaCl. För att avlägsna andra blodkroppar, användes en modifiering av Böyums metod såsom den beskrivits av Gambhir et al (1978). Den erhållna suspensionen lades på en Ficollösning och centrifugerades vid 400xg i 20 minuter vid 20°C. Efter centrifugeringen borttogs överfasen innehållande koksalt, Ficollösning, monocyter och granulocyter samt övre delen av erythrocyt-fasen. De kvarvarande röda blodkropparna suspenderades igen i fysiologisk NaCl och proceduren upprepades. Erythrocytkoncentratet späddes därefter med två delar buffert bestående av N-2-hydroxy-etyl-piperazin-N'-2-etansulfon syra (hepes) 50 mmol/L, trihydroxymetylaminometan (tris) 50 mmol/L, MgCl₂ 10 mmol/L, etylendiamintetra-ättiksyra 2 mmol/L, dextros 10 mmol/L, CaCl₂ 10 mmol/L, NaCl 50 mmol/L, KCl 5 mmol/L och 0,1% bovint serum albumin (pH 8,0 vid 23-25°C) och centrifugerades (10 minuter, 400xg, 4°C) varefter bufferten bortsögs och de röda blodkropparna späddes med lika stor volym av ovan nämnda hepesbuffert och antalet röda blodkroppar och retikulocyter bestämdes.

Bindningsstudier

Inkubation av röda blodkroppar med ¹²⁵I-insulin och separation av insulin bundet till dessa från fritt insulin utfördes enligt Gambhir et al (1978), dock med följande modifiering. Varje rör innehöll 400 µl av blodkroppssuspensionen, 40 picogram

^{125}I -insulin löst i 25 μl buffert och 100 μl innehållande en varierande mängd omärkt insulin, totalt 525 μl . Efter inkubation vid 15°C i 3,5 timmar lades 100 μl av inkubetet ovanpå, 100 μl buffert och 100 μl dibutylftalat (täthet 1,043) i kylda mikrocen-
trifugrör. Rören centrifugerades i en Beckman mikrofug B vid 4°C i 2,5 minuter. Rören skars ovanför de i botten packade röda blodkropparna. Radioaktiviteten hos dessa mättes därefter i en gammarräknare (Nuclear Enterprises NE 1600). Bindningen beräknades som maximal bindning vid lägsta insulinkoncentrationen (utan tillsats av omärkt insulin). Korrektion för ospecifik bindning baserades på aktiviteten i det rör som innehöll störst mängd omärkt insulin. Bindningsvärdet korrigerades därefter till $4,4 \times 10^{12}$ röda blodkroppar/L.

Metodfelet vid bindingsbestämning var 6,3% inom bestämmingar respektive 8,0% vid mätningar på samma patient två på varandra följande dagar.

Separation av röda blodkroppar

10 ml blod uppsamlades i heparinrör och fraktionerades enligt Danon och Marikovsky (1964). Blodet centrifugerades vid 20°C , 10 minuter och 400xg och plasman bortsögs. Kvarvarande röda blodkroppar blandades i en lika stor volym hepesbuffert (se ovan) med pH 8,0. Denna lösning lades ovanpå en ftalatlösning (täthet 1,096) och centrifugerades under 1 timme vid 15 000 varv/minut och 30°C i en Sörvall Superspeed RC 2-B med SS-34 rotor. Bufferten sögs bort och erytrocyterna med högre täthet togs i ett rör, och de med lägre täthet togs i ett annat. Vardera fraktionen löstes i fysiologisk NaCl och centrifugerades vid 20°C i 5 minuter, 400xg och NaCl sögs bort. På nytt uppslammades de röda blodkropparna i lika stor volym buffert. På en del av suspensionen bestämdes antalet röda blodkroppar, retikulocyter och pyruvatkinasaktivitet.

400 μl av blodkroppssuspensionen blandades med 100 μl hepesbuffert och 40 picogram ^{125}I -insulin löst i 25 μl buffert. Blandningen inkuberades över natten (21 timmar) vid 15°C . 100 μl av inkubetet lades ovanpå 100 μl buffert och 100 μl dibutylftalat

(täthet 1,043) i kyllda mikrocentrifugrör. Rören centrifugerades i en Beckman mikrofug B vid 4°C i 2,5 minuter. Därefter skars rören ovanför de i botten packade röda blodkropparna och radioaktiviteten mättes i en gammräknare (Nuclear Enterprises NE 1600). Bindningsvärdet korrigerades därefter till $4,4 \times 10^{-12}$ röda blodkroppar/L.

En jämförande studie av de två inkubationstiderna 3,5 och 21 timmar visade en förhöjning av medelvärdet med 29%, men att värdena inbördes korrelerade väl ($r=0,81$, $p<0,01$).

Insulinkänslighetstest

En venkateter lades in i en antecubital ven och en kontinuerlig infusion av fysiologisk NaCl påbörjades för att förhindra koagulering. Efter 40 minuter (= tiden 0) injicerades 0,012 IE insulin/kg kroppsvikt (Actrapid®). Denna insulinmängd motsvarar det tidiga insulinsvaret vid intravenös glukosbelastning (Thorell et al 1973). Före insulintillförseln togs tre blodprover vid -10, -5 och -1 minut för bestämning av insulin och blodsocker. Insulininjektionen gavs långsamt under 2 minuter, varefter blod togs för ytterligare blodsocker- och insulinbestämningar vid 5, 10, 20, 30, 45 och 60 minuter. Effekten av insulininjektionen mättes som skillnaden i blodsocker mellan ett medelvärde av de tre blodsockervärdena före injektionen och 20-minutersvärdet. Glukagon och tillväxthormon togs med bestämda intervall.

Glukostoleranstester

Vid intravenös glukosbelastning gavs glukos i form av 25%-ig lösning i dosen 0,5 g/kg kroppsvikt. Glukosens försvinnandehastighet uttrycktes i procent/minut, s.k. k_G . Vid oral glukosbelastning tillfördes glukos i form av en 10%-ig lösning i dosen 30 g/m² kroppsytta.

Övriga analyser

Blodsocker mättes (trippelmätningar) dels med en glukosoxidasmetod (G_G) (Marks 1959) och dels med en hexokinasetod (G_H) (Carroll et al 1970). Den linjära regressionen mellan metoderna var:

$$G_G = G_H \times 1,20 - 0,70$$

och blodsockerdata korrigerades med hänsyn härtill. Genom radioimmunoassay mättes insulin i plasma enligt Thorell och Larson (1978), glukagon i plasma enligt Faloona och Unger (1974) och tillväxthormon i plasma enligt Thorell och Larson (1978).

Röda blodkroppar räknades två gånger i ett Linson 431 celloskop och en gång i ett Linson 401 celloskop (Linson Instrument AB, Sverige). Medelvärde av dessa tre värden användes.

Retikulocyterna räknades vid två olika tillfällen enligt Atwaker och Erslev (1972) och medelvärde användes. Pyruvatkinasaktiviteten mättes såsom beskrivits av Amador och medarbetare (1965).

Erytrocyternas överlevnadstid mättes enligt standardmetod med ^{51}Cr (International Committee for Standardization in Haematology, 1980).

Betahydroxysmörtsyra i plasma bestämdes enligt Persson (1969) och $\text{HbA}_{1\text{C}}$ analyserades enligt Jeppsson och medarbetare (1978).

Statistiska metoder

Gruppresultat är uttryckt som medelvärde $\pm$ SEM. Linjär regressionsanalys, Student's t-test samt parat Student's t-test användes i delarbete I, II, III och VI. I delarbete IV användes Mann-Whitney-Wilcoxon's test mellan grupper och Wilcoxon's test för parade observationer. Spearmans rangkorrelation har dessutom utnyttjats i ramberättelsen (Hyrenius 1962).

C-PEPTID I URIN VID DEBUT AV DIABETES MELLITUS TYP I HOS BARN

Bakgrund

Humant C-peptid är en peptid som består av 31 aminosyror och som spaltas från proinsulin vid insulinets bildande. C-peptid och insulin utsöndras i ekvimolära mängder tillsammans med små mängder proinsulin och andra intermediärprodukter (Steiner et al 1969, Rubenstein et al 1969, Heding et al 1974, Horwitz et al 1975).

C-peptid, som inte tycks ha någon självständig biologisk betydelse (Kitabchi 1970, Yu & Kitabchi 1973, Hagen et al 1977), metaboliseras troligen ej eller i mycket ringa grad i levern (Stoll et al 1970, Polonsky et al 1983b, Polonsky et al 1984) i motsats till insulin varav cirka 50% binds vid varje leverpassage (Field 1973, Kaden et al 1973, Polonsky et al 1983a, Polonsky et al 1983b). C-peptid metaboliseras huvudsakligen i njurarna (Katz & Rubenstein 1973). C-peptid har en längre halveringstid än insulin. Dess metabola clearance är 4,4 ml/kg/min (Faber et al 1978), vilket är 2 1/2 - 10 gånger mindre än motsvarande värde för insulin (Sönksen et al 1973). Efter betacellsstimulering ökar koncentrationen i blod simultant av både insulin och C-peptid, men en diskrepans föreligger mellan de perifera koncentrationerna p.g.a. olikheter i fördelning och clearance.

Trots detta är serumkoncentrationerna av C-peptid och insulin starkt korrelerade både i portablod och perifert (Horwitz et al 1975). I njurarna extraheras både insulin och C-peptid genom glomerulär filtration. En mindre fraktion utskiljes med urinen, medan huvudparten reabsorberas i tubuli och degraderas i de peritubulära cellerna (Katz och Rubenstein 1973, Rabkin et al 1978). Cirka 5% av C-peptidproduktionen utsöndras i urin jämfört med cirka 0,1% av insulinproduktionen (Horwitz et al 1977). Närvaro av exogent insulin och/eller insulinantikroppar påverkar ej analysen av C-peptid. Det förhållande, att C-peptid utsöndras till relativt stor del i urin, och kunskapen om att utsöndringen vid oral glukosbelastning ökar parallellt med stegringen i plasmanivåerna (Meistas et al 1982), tyder tillsammans på att mätning av den totala C-peptidutsöndringen i urin kan användas som mått på patientens endogena insulinproduktion. Dygnsutsöndringen av

C-peptid torde också bättre avspegla insulinproduktionen än ett enstaka plasmaprov (Meistas et al 1981).

Man har diskuterat kvarvarande insulinsekretions prognostiska betydelse för remissionsfasen, och om man genom intensiv och snabb inställning av diabetespatienterna kan påverka remissionsperiodens längd och eventuellt förhindra en utmattnings av betacellerna.

Målsättning

- Genom att mäta dygnsutsöndringen av C-peptid i urin under första sjukdomsveckan och i begynnande remissionsfas värdera kvarvarande endogen insulinproduktion.
- Undersöka om graden av kvarvarande insulinproduktion har samband med barnets ålder vid debuten av diabetesjukdomen.
- Undersöka om det finns ett samband mellan kvarvarande insulinsekretion och parametrar som avspeglar den kortsiktiga prognosen.

Resultat och diskussion av delarbete I

Vid debuten av diabetes mellitus (dygn 1-6) kunde hos 18 av 19 barn påvisas mätbara nivåer av C-peptid i urin. Tolv av dem hade också mätbara nivåer i plasma. Medelvärdet för dygnsutsöndringen var högre ($7,11 \pm 1,22$ nmol/dygn/m² kroppsytta) hos diabetesbarnen än hos normala ($3,66 \pm 0,38$ nmol/dygn/m² kroppsytta, $p < 0,01$), medan däremot plasma C-peptidnivåerna var lägre ($0,14 \pm 0,03$ nmol/l) än hos kontrollerna ($0,32 \pm 0,02$ nmol/l, $p < 0,01$). Det funna medelvärdet för dygnsutsöndringen av C-peptid i urin överensstämmer med vad man tidigare rapporterat för normala barn och vuxna (Crossley et al 1981, Rappaport et al 1980, Horwitz et al 1977, Meistas et al 1981). Även de funna plasmanivåerna var i paritet med dem som rapporterats hos normala barn (Ludvigsson et al 1977) respektive vid debuten hos insulinkrävande diabetespatienter (Ludvigsson & Heding 1982, Madsbad et al 1982).

Vi fann ett samband mellan dygnsutsöndringen av C-peptid och C-peptidvärdena i blod ($r = 0,60$ för normala och $r = 0,68$ för

diabetespatienter, $p < 0,01$). Detta överensstämmer med resultaten i andra studier (Kuzuya et al 1978, Crossley et al 1981, Aurbach-Klipper et al 1983, Gerö et al 1983, Pasquali et al 1983). Vi kunde konstatera att plasmanivåerna av C-peptid hos diabetesbarnen endast var 1/3 av nivåerna hos normala barn, trots en av hyperglykemi maximalt stimulerad betacell, medan urinutsöndringen av C-peptid var nästan dubbelt så hög. Detta kan tolkas så att det renala läckaget för C-peptid är högre hos diabetesbarnen i insjukningsfasen än hos normala. Nyligen har Kolterman och Garvey (1984) visat att C-peptid clearance är förhöjd hos typ II diabetespatienter, vilket sålunda talar för att dessa har ett ökat renalt läckage av C-peptid. Även andra studier (Blix et al 1984) har antytt att C-peptidclearance kan ändras under fysiologiska omständigheter.

Vid förnyad studie 2-4 veckor senare noterades att värdet för dygnsutsöndringen av C-peptid låg på samma nivå som vid debuten. Korrelationen mellan de båda värdena var god ($r = 0,78$, $p < 0,01$). Mellan de båda undersökningstillfällena hade insulinbehovet minskat från $0,51 \pm 0,05$ till $0,37 \pm 0,05$ IE/kg kroppsvikt, ($p < 0,01$) och glukosutsöndringen hade minskat från 174 ± 29 mmol/dygn till 38 ± 12 mmol/dygn, ($p < 0,01$). Dessa resultat kan tyda på att en viss återhämtning av betacellens funktion hade skett, eftersom blodsockernivåerna, som stimulerar den endogena insulinproduktionen, torde ha varit betydligt lägre vid det senare tillfället.

Det relativt höga exogena insulinbehovet vid inkomsten, jämfört med det behov som föreligger senare under första månaden, samt en numerisk lika dygnsexkretion av C-peptid vid de två undersökningstillfällena, kan tyda på att det föreligger en initial insulinresistens. Mekanismen för en sådan minskad insulinkänslighet kan vara belägen på både receptor- och postreceptornivå. I detta sammanhang kan nämnas att i delarbete IV påvisades en minskad insulinbindning till erythrocyter hos nyinsjuknade barn med typ I diabetes. Insulinbindningen normaliserades i remissionsfasen. I delarbete VI påvisades en minskad insulinkänslighet i insjukningsfasen som också normaliserades i remissionsfasen.

Sambandet mellan urin C-peptidutsöndringen inom de första sex dagarna efter inkomsten till sjukhus och remissionsperiodens längd, HbA_{1c} efter ett halvt år och insulinbehovet efter 1 års behandling talar för att kvarvarande insulinsekretion har betydelse för den kortsiktiga prognosen. Våra resultat överensstämmer sålunda med retrospektiva studier hos barn med diabetes mellitus typ I, vilka visat att hög C-peptidsekretion korrelerar med längre remissionsperioder (Ludvigsson & Heding 1976, Knip et al 1982), lägre exogent insulinbehov (Rappaport et al 1980) och en bättre metabolisk kontroll (Ludvigsson et al 1977, Grajwer 1977, Dahlquist et al 1982a). Frånvaron av korrelation mellan HbA_{1c} i insjukningsfasen och såväl plasma som urin C-peptid kan tolkas så att HbA_{1c} inte reflekterar bukspottskörtelns funktionella status vid denna tidpunkt utan sannolikt återspeglar durationen av insulinbristen. Fyndet av en korrelation mellan urin C-peptidexkretionen initialt och insulinbehovet vid 12 månader, men avsaknad av korrelation med HbA_{1c} vid denna tidpunkt torde bero på att mer än kvarvarande betacellfunktion är viktig för den metabola kontrollen. Sålunda tycks effekten av låg kvarvarande endogen insulinsekretion kunna överskuggas av andra faktorer såsom förmågan att äta lämplig kost, graden av motion samt psykisk och fysisk stress.

Vissa indicier tyder på att diabetes mellitus typ I ej utgör en homogen sjukdomsgrupp. Sålunda synes barn som insjuknar vid låg ålder (före 5-7 års ålder) utgöra en speciell undergrupp. I denna åldersgrupp har rapporterats en högre frekvens hos pojkar (White & Graham 1971, Francois et al 1975, Bloom et al 1975, Dahlquist et al 1982b), avsaknad av årstidsvariation för diabetesdebuten (Bloom et al 1975, Dahlquist et al 1982b) samt en högre prevalens av diabetes i familjen (White & Graham 1971, Francois 1975). Våra resultat visar, att denna unga åldersgrupp dessutom kännetecknas av att de har en lägre nivå av C-peptid både i blod och i urin i insjukningsfasen, en kortare remissionsperiod och ett större insulinbehov/kg kroppsvikt efter 1 år. De yngre barnen synes således ha en mera uttalad betacellskada, när diagnosen ställs.

INSULINBINDNING TILL ERYTHROCYTER, METODOLOGISKA STUDIER

Bakgrund

Även om det vore önskvärt att undersöka insulinreceptorer på klassiska målorgan för insulin såsom muskel-, lever- och fettceller har den kliniska forskningen, med undantag för fettceller (Olefsky et al 1974), begränsats till mer lättillgängliga cellmodeller såsom monocytter (Schwartz et al 1975), granulocyter (Fussganger et al 1976), erythrocyter (Gambhir et al 1978), trombocyter (Hajek et al 1979) och placentamembraner (Harrison et al 1977). Av dessa är erythrocyter, monocytter och fettceller de mest studerade celltyperna. Studier av insulinreceptorer på fettceller har åtskilliga fördelar, då de tillåter samtidig mätning av insulinbindning till receptorn och insulineffekt. Nackdelen med fettcellsmodellen är att den kräver öppen biopsi för framskaffande av den mängd fettvävnad som behövs (cirka 2 g). Då öppen biopsi är ett invasivt ingrepp, krävs lokalanestesi, och det har ifrågasatts om detta kan påverka bindningen till receptorn (Pedersen 1983). Stora fettceller är också sköra och faller lätt sönder, vilket gör att det kan vara svårt att kvarhålla ett stationärt läge under fysiologiska temperaturbetingelser (Olefsky et al 1974). Detta förhållande, sammantaget med att det föreligger skillnader i fettcellstorlek även inom samma region (Pedersen et al 1981c, Pedersen et al 1982b), begränsar starkt möjligheten till kontrollstudier av t.ex. terapieresultat. Den icke-specifika bindningen är emellertid låg och metoden har en hög precision vid upprepade bestämningar (Pedersen et al 1981c).

Användningen av monocytter och erythrocyter vid receptorstudier har den fördelen att longitudinella studier kan göras. Dock är blodmängden vid monocytstudier (50-120 ml) betydande, vilket begränsar dess användning om patientmaterialet är barn. Vid preparation erhålles 10-30% monocytter, 70-90% lymfocyter och cirka 1% utgörs av granulocyter (samt ett varierat antal trombocyter och erythrocyter) (Schwartz et al 1975, Beck-Nielsen et al 1977). Av den totala insulinbindningen svarar dock monocytterna för 80-90% (Schwartz et al 1975, Beck-Nielsen et al 1977), varför man brukar

beteckna hela den uppmätta bindningen som monocyttbindning. Bindningsstudierna görs vid 15-22°C eftersom det vid 37° är svårt att erhålla "steady state" (jämvikts)-förhållande p.g.a. en uttalad insulindegradation (Beck-Nielsen & Pedersen 1979), och vanligtvis mäts insulinbindningen vid pH 8, eftersom ett bindningsoptimum föreligger där (Pedersen & Beck-Nielsen 1976). Studierna göres sålunda ej under helt fysiologiska temperatur- och pH-betingelser till skillnad från fettcellsstudierna. I jämförelse med andra receptorbestämmningar är monocyt-receptormetoden behäftad med en relativt hög ospecifik bindning och har en låg precision vid upprepade bestämningar (Pedersen et al 1980).

För erythrocytreceptorstudier åtgår en betydligt mindre mängd blod, nämligen cirka 10 ml, vilket är en mängd som kan accepteras även vid studier på barn. Analyserna utföres även här under icke fysiologiska betingelser, då temperaturen är 4-22°C och pH 8,0 med samma motivering som gäller monocytstudierna (Gambhir et al 1981b). Den ospecifika bindningen är vid ovannämnda temperatur dock relativt låg och precisionen är hög vid upprepade bestämningar (Pedersen et al 1980), vilket överensstämmer med våra studier.

Hos humana fettceller, monocyter och erythrocyter finns egenskaper som är karaktäristiska för receptorbinding, d.v.s. specificitet för insulin, hög affinitet, pH- och temperaturberoende (Gavin III et al 1972, Olefsky et al 1974, Pedersen & Beck-Nielsen 1976, Gambhir et al 1978). Egenskaperna hos ovan nämnda insulinreceptorer delas av insulinreceptorer på fett-, muskel- och leverceller hos djur (Soll et al 1974, Le Marchand-Brustel et al 1978).

Vid studier av kaniner hos vilka man inducerat hemolytisk anemi, fann Thomopolous och medarbetare (1978) en minskning av insulinbindningen i och med erythrocyternas utmognad och retikulocytosens försvinnande. Frågan som då uppstod var vilket inflytande erythrocyternas åldrande har på insulinbindningen. Kan erythrocytreceptormodellen tillämpas vid studier både på patienter med både normal respektive ökad erytropoes? Påverkar variation av retiku-

locytantalet inom normalområdet resultatet vid insulinbindningsstudier?

Målsättning

- Att undersöka om omogna erythrocyter hos människan har ökad insulinbindning jämfört med mogna erythrocyter.
- Att undersöka åldersfaktorns relevans vid användande av erythrocytreceptormodellen för studie av insulinbindningen.

Resultat och diskussion av delarbete II och III

Mot bakgrund av Thomopolous' och medarbetares (1978) ovannämnda studie valde vi att studera insulinbindningen till erythrocyter hos patienter med stor variation i retikulocytantal. Ett starkt positivt samband kunde då påvisas mellan insulinbindning och retikulocytantal ($r=0,85$, $p<0,01$). Liknande resultat rapporterades ungefär samtidigt från flera olika forskargrupper (Kosmakos et al 1980, Eng et al 1980, Dons et al 1981a, Ward et al 1981, Ivarsson & Thorell 1981). I en normalpopulation kunde däremot inte någon korrelation påvisas varken mellan insulinbindning och pyruvatkinasaktivitet, en annan välkänd åldersparameter (Piomelli et al 1968) hos erythrocyterna, ($r=-0,39$, $p>0,05$), eller retikulocytantal ($r=-0,15$, $p>0,05$). Frånvaron av ett sådant samband torde bero på den obetydliga variation i antalet retikulocyter som kännetecknar en normalpopulation. Brist på ett sådant samband har konstaterats även i andra studier (Kappy & Plotnick 1979, Puukka et al 1983). För att undersöka huruvida insulinreceptorer på erythrocyter är begränsade till enbart retikulocyter, vilket föreslagits, gjordes en separationscentrifugering genom ett täthetsmedium. Eftersom de yngre röda blodkropparna är lättare, ansamlas dessa ovanför täthetsmediet. Efter separationen återfanns i överfasen ett ökat antal retikulocyter, en ökad pyruvatkinasaktivitet samt en högre insulinbindning. Kvoten mellan överfas och underfas var för insulinbindning 1,5 och var för pyruvatkinasaktivitet i samma storleksordning, medan kvoten vad gäller retikulocytantal varierade mellan 3,4-4,6. Genom detta förfaringssätt återkom sambandet mellan åldersparametrar och insulinbindning till erythrocyter även

Tabell 1

Variation vid bestämning av insulinbindning till erythrocyter

Total varians	$4,6^2$	(100%)
Variansens delkomponenter		
Intraassay error	$0,7^2$	(2%)
Interassay error	$0,8^2$	(3%)
Regression på pyruvatkinasaktivitet	$0,59^2 \times 4,6^2$	(35%)
Övriga komponenter	$3,56^2$	(60%)

inom en normalpopulation (insulinbindning/pyruvatkinasaktivitet $r=0,59$, $p<0,01$, insulinbindning/retikulocytantal $r=0,44$, $p<0,01$). Anledningen till detta är att separationen förstärker effekten av åldersfaktorerna.

Av den totala variansen vad avser insulinbindning till erythrocyter visar det sig att den metodologiska variationen svarar för cirka 5%, åldersrelationen svarar för 35%, medan återstående, dvs cirka 60%, är oberoende av dessa faktorer (tabell 1). Detta innebär att faktorer oberoende av den röda blodkroppens ålder har ett relativt stort inflytande, och inte förrän åldersfaktorerna överskrider det normala, såsom vid hemolytiska tillstånd, kommer deras inflytande att öka till en sådan grad att de överskuggar effekten av icke-åldersberoende faktorer. Ward och medarbetare (1981) har emellertid föreslagit, att en korrektionsfaktor borde införas så att bindningen korrigerades med hänsyn till antalet retikulocyter. Camagna och medarbetare (1983) förde samma resonemang vad avser pyruvatkinasaktiviteten. Vi har beräknat hur de ovan föreslagna korrektionerna påverkar bindningsvärdena i vårt material: Spearmans rangkorrelation mellan bindningsvärden före och efter korrektion var för pyruvatkinas $r=0,92$ ($p<0,01$) och för retikulocytantal $r=0,97$ ($p<0,01$) (tabell 2). Hos individer med normal erytropoes behöver man sålunda inte introducera någon korrektionsfaktor för retikulocytantal eller pyruvatkinasaktivitet.

Även hos personer med ökad erytropoes visade bindningen en nära relation till både retikulocytantal ($r=0,69$, $p<0,01$) och pyruvatkinasaktivitet ($r=0,65$, $p<0,05$). Under första månaden av normalisering av erytropoesen visade sig minskningen av retikulocytantalet vara betydligt snabbare ($T\ 1/2 = 9$ dagar) än nedgången av insulinbindningen ($T\ 1/2 = 37$ dagar) och pyruvatekinaseaktiviteten ($T\ 1/2 = 36$ dagar). Dons och medarbetare (1981a) har visat att insulinreceptorkoncentrationen minskar kontinuerligt som en exponentiell funktion av erythrocytens åldrande. Vi kunde genom att studera försvinnandet av ^{51}Cr -märkta erythrocyter hos en patient med hereditär sfärocytos som genomgick splenektomi visa, att insulin är bundet också till äldre erythrocyter. Antalet ^{51}Cr -märkta erythrocyter minskade nästan parallellt med värdet

för insulinbindningen och dessa parametrar nådde ett jämviktsläge betydligt senare än antalet retikulocyter.

Resultaten ovan tyder på att receptorn hos den mogna erytrocyten försvinner gradvis med åldrandet. Den mogna erytrocyten torde ej kunna modifiera sin koncentration av insulinreceptorer, eftersom den proteinsyntes, som behövs för "upp"- och "nerreglering" av receptorn, är begränsad mot bakgrund av att erytrocyten saknar kärna (Gavin III et al 1974, Blackard et al 1978, Kosmakos & Roth 1980).

Det är därför sannolikt att antalet insulinreceptorer på erytrocyten fastställs under dess utvecklingsfas. Om så är fallet och åldrandet sker med konstant hastighet, torde erytrocytens insulinbindning kunna reflektera det metabola tillstånd som föreligger under dess tidiga utvecklingsstadier. Erytrocyternas långa överlevnadstid (cirka 120 dagar) gör dem ej lämpliga som indikatorer på kortsiktiga förändringar (jämför monocyternas överlevnadstid på timmar till dagar). Emellertid torde kroniska metabola förändringar påverka den genomsnittliga receptorkoncentrationen hos erytrocytpopulationen.

Tabell 2

Insulinbindning till erythrocyter, före och efter normalisering till medianvärdet för retikulocyttantal ($10 \times 10^9/L$) respektive pyruvatkinasaktivitet (2,4 U/L erythrocyter)

	Bindning före normalisering %	Bindning efter normalisering till retikulocyttantal %	r_S^*	P	Bindning efter normalisering till pyruvatkinasaktivitet %	r_S^*	P
Vuxna	15.6 $\pm$ 0.6	15.3 $\pm$ 0.6	0.92	<0.01	13.8 $\pm$ 0.7	0.67	<0.05
Barn	20.3 $\pm$ 1.0	20.2 $\pm$ 1.0	0.95	<0.01	21.3 $\pm$ 1.2	0.90	<0.01
Totalt	17.9 $\pm$ 0.8	17.8 $\pm$ 0.8	0.97	<0.01	17.5 $\pm$ 1.1	0.92	<0.01

* r_S = rangkorrelation mellan bindningsvärde före och efter normalisering

INSULINBINDNING TILL ERYTHROCYTER HOS NYINSJUKNADE BARN MED
DIABETES MELLITUS TYP I OCH I ETT FALL AV DIABETES MELLITUS
KOMBINERAT MED BINJUREBARKSINSUFFICIENS

Bakgrund

De metodologiska delarbetena II och III visade, att det borde vara möjligt att använda insulinbindningen till erythrocytreceptorn som uttryck för receptorförändringar vid kroniska tillstånd. Är då förändringar i insulinbindning till erythrocyter representativa även för andra receptormodeller? Man har vid jämförande studier av insulinbindning till fettceller, monocyter och erythrocyter hos normala personer inte kunnat visa ett likartat reaktionsmönster mellan de olika celltyperna inom varje individ (Pedersen et al 1982b), däremot fanns ett samband hos typ I diabetespatienter mellan fettceller och erythrocyter (Pedersen & Hjøllund 1982), medan inget samband förelåg hos dessa patienter mellan fettceller och monocyter respektive erythrocyter och monocyter (Pedersen och Hjøllund 1982). Hos patienter med insulinresistent diabetes i kombination med acanthosis nigricans har man funnit en parallell reduktion i insulinbindningen till fettceller, monocyter och erythrocyter (Pedersen et al 1981b) liksom till fibroblaster, monocyter och erythrocyter (Rüdiger et al 1983). Vid obesitas har man funnit ett samband vad gäller insulinbindningen mellan fettceller och monocyter (Olefsky 1976a). Vid jämförelse mellan erythrocyter och monocyter har man funnit en korrelation mellan insulinbindningen vid vissa tillstånd (Pedersen et al 1980, Ward et al 1981, Dons et al 1981b, Spanheimer et al 1982, Briggs et al 1981, Kobayashi et al 1980, Wachslight-Rodbard et al 1979, Wachslight-Rodbard et al 1981), medan man vid andra tillstånd ej har kunnat påvisa något sådant samband (DePirro et al 1980b, Pedersen et al 1981a, Tsibris et al 1980, Pedersen et al 1982c).

Det är alltså kontroversiellt om det existerar ett samband mellan insulinbindningen till de olika celltyperna. Anledning till olikheter kan bero dels på att cellerna har olika överlevnadstid och dels på att expositionen för hormonet varierar mellan olika organ och även att olika metabola situationer såsom acidosis, glukokorti-

koidnivå etc. kan påverka insulinbindningen olika. Sammantaget kan studierna ovan möjligen tyda på att kroniska tillstånd påverkar de olika cellernas insulinreceptorer likartat, medan detta ej är fallet vid akuta förändringar.

Då hyperinsulinemi ger en insulinresistens och en "nerreglering" av antalet insulinreceptorer (Olefsky 1976a, Bar et al 1976), har man antagit att insulinbrist skulle medföra en "uppreglering" av antalet insulinreceptorer och en ökad insulinkänslighet. Emellertid har insulinbindningen till erythrocyter ej studerats vid det insulinbristtillstånd som föreligger hos nyinsjuknade, obehandlade barn med typ I diabetes mellitus.

Målsättning

- Att studera insulinbindningen till erythrocyter hos barn med diabetes mellitus i samband med debuten innan någon insulinterapi påbörjats.
- Att studera om insulinbindningen förändras efter det att insulinterapi har påbörjats och patienten gått in i en remission.
- Att studera insulinbindningen vid fall av koexisterande diabetes mellitus och binjurebarksinsufficiens före och efter steroidsubstitution.

Resultat och diskussion av delarbete IV och V

^{125}I -insulinbindningen till erythrocyter hos nyinsjuknade barn med typ I diabetes mellitus var något lägre ($10,4 \pm 0,4\%$) än hos normala barn ($11,6 \pm 0,5\%$, $p < 0,05$). Efter 3-6 veckors insulinbehandling hade bindningen ökat till normal nivå ($12,1 \pm 0,4\%$).

Detta överensstämmer med andra studier som har visat att

^{125}I -insulinbindningen till erythrocyter är normal hos behandlade diabetiker (Kappy & Plotnik 1979, Pedersen et al 1982a, Nosadini et al 1982). Pedersen och medarbetare (1982a) fann i en grupp av obehandlade, huvudsakligen vuxna, insulinberoende diabetespatienter en större variation i insulinbindningen till erythrocyter än hos normala men ett normalt medelvärde. P.g.a. den

stora variation indelades dessa patienter arbiträrt i två subpopulationer, "hög"- och "låg"-bindare. De förra hade högre plasmaketonvärden och lägre plasmainsulinvärden än "lågbindarna". Vid motsvarande indelning av vårt material fann vi ingen skillnad mellan "högbindare" och "lågbindare" vad avser de perifera nivåerna av insulin, C-peptid, blodsocker och betahydroxysmörtsyra. Det tycks emellertid som om våra patienter var betydligt mindre metabolt derangerade och sålunda närmast motsvarar Pedersens "lågbindare". Normaliseringen av den sänkta insulinbindningen i vår studie är då helt i linje med vad som skedde med "lågbindargruppen" i Pedersens och medarbetares studie.

Motstridiga resultat har publicerats vad avser glukokortikoidnivåernas betydelse för insulinbindningen. Såväl kliniskt som experimentellt finns åtskilliga indicier på att binjurebarksinsufficiens åtföljs av en ökad insulinkänslighet, medan tillstånd med hyperkortisolemi kännetecknas av insulinresistens. Bakomliggande mekanismer härför är emellertid inte klarlagda ännu. Hos normala individer har administrering av prednison rapporterats ge en ökning av antalet receptorer på monocyter (Beck-Nielsen et al 1980) medan tillförsel av dexametason och prednison i studier på erytrocyter (Yasuda & Kitabchi 1980) och dexametason och kortison på monocyter (DePirro et al 1980a), har visats ge en minskning av insulinbindningen. Två dagar innan en patient med diabetes mellitus typ I utvecklade en incipient Addisonkris, hade vi tillfälle att studera hans insulinbindning till erytrocyterna. Denna var låg (6,9%) trots ett sedan en tid tillbaka sjunkande insulinbehov. Efter steroidsubstitution måste den tillförda insulinmängden fördubblas och man fann då också en normalisering av insulinbindningen till erytrocyterna (12,0%).

Enligt gängse teorier om insulinreceptorkoncentrationen skulle låga insulinnivåer medföra en ökad receptorkoncentration och högre insulinbindning. Man har också funnit höga receptorantal i enlighet med förväntad "upp-reglering" vid hypoinsulinämiska tillstånd såsom anorexia nervosa (Wachslight-Rodbard et al 1979) och pankreasinsufficiens (Nosadini et al 1982). Emellertid är inte alla hypoinsulinämiska tillstånd förenade med en ökad insulinbindning.

Vid tillväxthormonbrist har rapporterats oförändrad insulinbindning, trots att detta tillstånd kännetecknas av en reduktion i mängden cirkulerande insulin (Lippe et al 1981).

Orsaken till utebliven "upp-reglering" av insulinreceptorerna hos såväl obehandlade patienter med typ I diabetes mellitus som i ovan nämnda fall av diabetes mellitus och koexisterande Morbus Addison är oklar. Möjligen beror den ökade insulinkänsligheten vid binjurebarksinsufficiens inte på förändringar på receptornivå utan på postreceptornivå. Den minskade insulinbindningen till erytrocyterna före steroidbehandling kan i så fall tänkas vara en fysiologisk justering av receptorn med anledning av förändringar på postreceptornivå. Den sänkta insulinbindningen, som förelåg före behandling av insulinbristen hos barn med diabetes typ I, kan analogt bero på en postreceptorstörning som förhindrar den normala "upp-regleringen". Normaliseringen av bindningen sedan det diabeteska tillståndet korrigerats stöder denna teori. Nyligen har rapporterats förekomst av autoantikroppar riktade mot insulinreceptorer hos nyinsjuknade diabetespatienter (Maron et al 1983). Sådana autoantikroppar kan tänkas bidra till den sänkta insulinbindningen.

INSULINKÄNSLIGHET HOS NYINSJUKNADE BARN MED DIABETES MELLITUS TYP I

Bakgrund

Det är fortfarande oklart vilka mekanismer som påverkar förloppet under insjukningsfasen av diabetes mellitus. Vilken roll spelar andra faktorer än den direkta insulinbristen? Redan Himsworth rapporterade (1932, 1936), som tidigare nämnts, att insulin varierade i sin verkan mellan olika patienter med diabetes mellitus, vilket talar för att inte bara avsaknad av insulin är av betydelse. Det har sålunda diskuterats, om resistens mot insulinets effekt kan vara en orsak till denna varierande verkan eller på något sätt kan bidra till försämrad metabolisk kontroll. Vad gäller typ II diabetes är man nu ense om att insulinresistens föreligger (Reaven & Olefsky 1978, Olefsky 1981). Vid diabetes av typ I däremot är tillgängliga data mer osäkra. Sålunda har man påvisat både normal insulinkänslighet (Martin & Stocks 1967, Ginsberg 1977, Park et al 1974, Thorell et al 1975) och perifer resistens (Martin & Stocks 1967, Ginsberg 1977, Butterfield & Whichelow 1965, Zampa et al 1967, Simonson et al 1981).

Insulinkänsligheten in vivo kan mätas med ett antal olika metoder. Den av Rabinowitz och Zierler (1962) använda metoden går ut på att mäta insulinets förmåga att stimulera glukosupptaget i perfunderad underarm. En enklare metod att beräkna insulinets in vivo-verkan beskrevs av Alford och medarbetare (1971), det så kallade insulin-toleranstestet (ITT), som innebär att 0,1 IE insulin/kg kroppsvikt injiceras intravenöst. Detta sätt att undersöka insulinets in vivo-verkan har åtskilliga begränsningar. Den initiala plasma-insulinkoncentrationen är ofysiologiskt hög och medför en påtaglig minskning av glukoskoncentrationen, varvid hormoner, som motverkar effekten av insulin, frisättes (Rizza et al 1979).

Ovannämnda studier av Himsworth, Rabinowitz och Zierler samt Alford och medarbetare är dynamiska studier som genomföres då plasmaglukos- och plasmainsulinkoncentrationerna inte är i ett "steady state" (jämvikts)-läge. För att göra det möjligt att

experimentellt jämföra glukosutnyttjandet vid identiska insulinstimuli under "steady state"-betingelser har olika s.k. "clamp"-tekniker införts. Vid insulinsuppressionstestet (IST) infunderar man konstanta mängder av insulin, glukos, adrenalin och propranolol (Shen et al 1970). Denna metod för att bestämma insulinets in vivo-verkan innefattar sålunda infusion av adrenalin och propranolol för att blockera den endogena insulinsekretionen. Möjligheten finns emellertid att dessa farmaka interfererar med insulinets verkan (Altszuler et al 1967, Shikama & Ui 1975, DeFronzo et al 1979).

För att undvika detta problem har adrenalin och propranolol i andra studier ersatts med somatostatin (Harano et al 1977). Detta hormon hämmar förutom den endogena insulinsekretionen också glukagon- och tillväxthormonsekretionen (Gerich et al 1974). Samma invändning, som riktats mot användande av adrenalin och propranolol, gäller därför även för somatostatin (Sacca et al 1979).

Den ytterligare modifikation av "glukos-insulin clamp"-metoden, som utarbetats av Andres och medarbetare (Sherwin et al 1974), har avsevärda teoretiska fördelar. Metoden bygger på användandet av en negativ "feed back" algoritm för att kvantitera mängden intravenös glukos, som behöver tillföras för att kvarhålla den basala glukoskoncentrationen oförändrad under en konstant insulininfusion (s.k. euglukämisk clamp). Denna metod är mer komplicerad än IST-metoden men undviker användandet av potenta farmakologiska medel som kan komplicera tolkningen av resultaten. Dock föreligger svårigheter i att kvarhålla blodsockerhalten på oförändrad nivå under hela "clamp"-perioden (Doberne et al 1981).

En av de funktionella förändringar som tidigast kan påvisas under utvecklingen till manifest diabetes mellitus typ I är en minskning av det tidiga insulinsvaret vid intravenös glukosbelastning (Simpson et al 1968, Srikantha et al 1984). Det är möjligt att förändringar i effekten av insulin på glukosutnyttjandet vid debuten kan återfinnas i första hand i svaret på akuta för-

ändringar i insulinnivåerna snarare än under "steady state"-förhållanden. Vi har därför studerat insulinkänsligheten med hjälp av en experimentell modell, som mäter förändringar i blodsockernivån efter en insulininfusion som simulerar det tidiga fysiologiska insulinsvaret. Den inducerade blodsockersänkningen är av sådan storleksordning att hormoner med motreglerande effekt ej stimuleras till ökad insöndring.

Målsättning

- Att studera insulinkänsligheten i insjukningsfasen hos barn med typ I diabetes mellitus.
- Att studera om insulineffekten varierar med svårighetsgraden av det diabetiska tillståndet.

Resultat och diskussion av delarbete VI

Den givna insulininfusionen (0,012 IE/kg kroppsvikt) ökade plasmainsulinnivåerna till jämförbar nivå hos diabetespatienter och kontroller. Hos normala barn gav detta ett blodsockerfall från $4,6 \pm 0,1$ mmol/L till ett minimum vid 20 minuter på $3,6 \pm 0,1$ mmol/L, vilket motsvarar en minskning på $20,9 \pm 2,1\%$ (range 10-40%) och blodsockret återvände därefter till ursprungsvärdet vid 60 minuter.

I diabetesgruppen orsakade samma insulinmängd en sänkning av blodsockernivån från $11,3 \pm 1,0$ mmol/L till $10,2 \pm 1,1$ mmol/L vid 20 minuter motsvarande en minskning på $14,3 \pm 3,5\%$ (range 0-53%) och blodsockernivån kvarlåg därefter oförändrad.

Svårighetsgraden av det diabetiska tillståndet varierade mellan de olika patienterna. Effekten av insulininfusionen visade sig vara korrelerad till graden av rubbad glukosomsättning mätt med olika parametrar såsom fastebloodsocker, värde på k_g vid intravenös glukostoleranstest och blodsockernivån vid 60 och 120 minuter vid den orala glukosbelastningen (se tabell 3).

Tabell 3

Korrelation mellan blodsockersänkning vid insulinkänslighetstest och fasteblodsocker respektive resultaten vid intravenös och oral glukosbelastning med användande av dels absolut dels relativ blodsockersänkning

	Blodsockersänkning			
	Absolut		Relativ	
	r	p	r	p
Fasteblodsocker	-0,51	<0,05	-0,79	<0,01
k _G IVGTT	0,62	<0,01	0,71	<0,01
60 min. blodsocker vid OGTT	-0,60	<0,05	-0,69	<0,01
120 min. blodsocker vid OGTT	-0,63	<0,01	-0,76	<0,01

Vid närmare analys av data som avspeglar svårighetsgraden av det diabetiska tillståndet utkristalliserade sig två grupper av patienter. Den ena gruppen kännetecknades av fasteblodsocker under 8 mmol/L, ett signifikant högre k_G vid intravenös glukosbelastning samt ett signifikant lägre blodsocker vid 60 resp. 120 minuter vid oral glukosbelastning. Blodsockersänkningen efter insulininfusionen hos denna grupp uppvisade samma mönster som hos normala och var $2,0 \pm 0,4$ mmol/L eller $36,2 \pm 6,0\%$ (range 18-53%). Denna sänkning var t.o.m. större än hos normala (20,9%, $p < 0,05$). Insulineffekten i den andra gruppen, som kännetecknades av ett mera uttalat diabetiskt tillstånd, var klart reducerad. Blodsockersänkningen var $0,7 \pm 0,1$ mmol/L eller $5,6 \pm 0,8\%$ (range 0-11%). Skillnaden i blodsockersänkning mellan dessa båda grupper understryker ytterligare sambandet mellan insulineffekt och graden av insulinbrist och därav resulterande metabola störning.

Tidigare studier över insulinkänsligheten vid typ I diabetes mellitus har givit divergerande resultat. Både insulinresistens och normal känslighet har sålunda rapporterats. Vi har eftergranskat dessa studier och därvid relaterat den funna insulin-effekten till svårigheten av det diabetiska tillståndet med blodsockernivån som indikator därpå. I studierna som visade en markerad insulinresistens var blodsockerkoncentrationen hög (Butterfield & Wichelow 1965, Zampa et al 1967, Simonson et al 1981). Studien av Martin och Stocks tyder på en tendens till insulinresistens hos patienter med hög fasteblodsockerhalt, men normal insulinkänslighet vid lägre fasteblodsockerkoncentration. I andra studier har man funnit normal insulinkänslighet hos diabetespatienter med väsentligen normala fasteblodsockernivåer (Park et al 1974, Thorell et al 1975) och i Ginsbergs studie (1977) hade nio patienter normal insulinkänslighet och de tre patienter som var insulinresistenta var de med fasteblodsockerkoncentration över 10 mmol/L.

Det ska framhållas att en ökad blodsockerkoncentration per se inte är associerad med nedsatt insulinkänslighet. Detta har visats med den teknik som används i denna studie (Thorell 1973) och med användandet av flera andra metoder (Best et al 1981, DeFronzo et al

1982). I studie på hund kunde Cherrington och medarbetare (1978) vidare visa att försvinnandehastigheten för glukos ökade proportionellt med ökningen av plasmaglukosnivån.

Fem av patienterna återtestades i remissionsperioden. Hos fyra av dem, som initialt var påtagligt hyperglykämiska och hade nedsatt insulinkänslighet, noterades nu normal känslighet (blodsockersänkningen $0,7 \pm 2$ mmol/L eller 6%, respektive $2,0 \pm 0,2$ mmol/L eller 23%). Ett barn med fasteblodsocker på 4,5 mmol/L vid de initiala testen uppvisade normal känslighet vid de båda testtillfällena (31% respektive 25%). Dessa resultat tyder åter igen på att insulinresistens inte är associerat till diabetes mellitus typ I per se, utan är ett sekundärt fenomen.

Den initiala insulinresistensen kan sannolikt bero på förändringar belägna både på receptor- och på postreceptornivå. En minskad insulinreceptorkoncentration anses bidra till den insulinresistens som påvisats vid obesitas (Olefsky 1976b), uremi (Gambhir et al 1981a) och akromegali (Muggeo et al 1979). Den sänkta insulinbindningen, som påvisades i delarbete IV hos nyinsjuknade diabetespatienter, kan tyda på att ett minskat receptortantal kan ha betydelse för utvecklingen av nedsatt insulinkänslighet. Emellertid fann vi i nämnda delarbete inget samband mellan insulinbindningen och graden av metabolisk störning. Randle och medarbetare (1963) hävdade utifrån sina studier, att hög koncentration av fettsyror och ketonkroppar kan interferera med glukosmetabolismen och medföra insulinresistens. I vår studie påvisades nedsatt insulinkänslighet även hos patienter utan ketonuri, vilket tyder på att sådan kan utvecklas, även om det inte föreligger en uttalad ketoacidosis. Det återstår att klarlägga, vilka relativa bidrag som förändringar på receptor- respektive postreceptornivå har till den minskade insulinkänsligheten.

SAMMANFATTNING OCH KONKLUSION

Avsikten med denna studie var att undersöka olika mekanismer, som kan ligga bakom den bristande insulinverkan som föreligger i samband med insjuknandet i diabetes mellitus typ I hos barn. Som ett mått på kvarvarande insulinproduktion studerades C-peptidutsöndringen i urin. Erytrocytmodellens tillämpbarhet för studier av insulinbindning undersöktes ur aspekten hur erytrocytens åldrande påverkar insulinbindningen. Insulinbindningen till erytrocyter som ett mått på insulinreceptormängden studerades hos nyinsjuknade barn med diabetes mellitus före insatt behandling och i remissionsfas. Insulinkänsligheten undersöktes i insjukningsfasen och relaterades till parametrar som avspeglar det diabetiska tillståndet.

1. Jämfört med normala barn uppvisar barn med diabetes mellitus vid debuten en ökad utsöndring av C-peptid i urin. Denna höga utsöndring var av samma storleksordning 1-3 veckor senare i samband med begynnande remission. Övervägande delen av patienterna hade vid inkomsten innan insulinterapi initierats mätbara, men låga C-peptidnivåer i plasma. Sammantaget talar resultaten för att det i initialfasen av diabetes mellitus typ I föreligger ett ökat läckage av C-peptid i njurarna.
2. C-peptidutsöndringen i urin som mått på kvarvarande insulinsekretion visade ett positivt samband till remissionsperiodens längd och ett negativt samband till insulinbehovet och HbA_{1C} under första sjukdomsåret. Sålunda är kvarvarande insulinsekretion av betydelse för prognosen på kort sikt.
3. Barn som vid diabetesdebuten var under 7 år hade en lägre C-peptidutsöndring jämfört med äldre barn. Dessa barn torde ha utvecklat en mera uttalad betacellskada när diagnosen ställdes. Remissionsperioden uteblev eller var betydligt kortare än jämfört med äldre barns och de hade ett större insulinbehov under första sjukdomsåret. Möjligen utgör de en undergrupp av barn med diabetes mellitus typ I.

4. Vid ökad erytropoes visade insulinbindningen en nära relation till både retikulocytantal och pyruvatkinasaktivitet. Unga erythrocyter hade en högre insulinbindning än äldre. Vid sjukdom med ökad erytropoes är därför erythrocytmodellen olämplig för studier av insulinbindning.
5. Resultaten talade för att vid normal erytropoes är förändringar i ^{125}I -insulinbindningen till erythrocyter till övervägande del icke åldersberoende eller beroende på metodologiska faktorer. Man kan därför anta att förändringar i insulinbindningen till erythrocyter avspeglar andra, troligen metabola, faktorer.
6. Vid diabetesdebuten, innan insulinbehandling påbörjats, förelåg en sänkt ^{125}I -insulinbindning till erythrocyter. Detta förhållande kan bidra till den initiala insulinresistensen, men sannolikt torde även andra faktorer ha betydelse härför. Insulinbindningen normaliserades i remissionsperioden.
7. En patient med typ I diabetes mellitus, som utvecklade Morbus Addison, uppvisade före substitution med glukokortikoid och mineralokortikoid en sänkt insulinbindning till erythrocyter. Detta kan tala för att den ökade insulinkänslighet som föreligger vid hypokortisolemi beror på förändringar på post-receptornivå. Efter insatt behandling normaliserades bindningen.
8. Hos nyinsjuknade barn med diabetes konstaterades ett samband mellan blodsockersänkningen efter tillförsel av fysiologisk insulindos och svårighetsgraden av det diabetiska tillståndet (värderat utifrån fasteblodsocker, k_G vid intravenös och blodsocker vid 60 och 120 minuter vid oral glukosbelastning).
9. Vid undergruppering av barnen med hänsyn tagen till svårighetsgraden vid det diabetiska tillståndet visade det sig, att de med mindre uttalad metabolisk störning hade en normal eller t.o.m. ökad insulinkänslighet. De med mer uttalad metabolisk störning hade påtagligt nedsatt insulinkänslighet. Barn som återsundersöktes i remissionsfasen visade normal insulin-känslighet.

Summary in English

The purpose of this investigation was to study various mechanisms which may explain the impaired insulin effect, which characterize the initial phase of type I diabetes mellitus. Thereby we have studied the C-peptide excretion, the insulin binding to the erythrocytes and the insulin sensitivity in children with newly detected type I diabetes mellitus and restudied them in some cases in the remission phase.

In the children with diabetes the C-peptide excretion was higher while the plasma C-peptide level was substantially lower as compared with normal controls. These findings indicate a higher urinary leakage of C-peptide at this phase of the disease. Follow-up studies indicated that the higher the level of the urinary C-peptide at admission, the longer the remission period and the less the insulin requirement after one year. The urinary C-peptide excretion was in the same range when reexamined 2-4 weeks after hospitalization of the children at a time when they had entered a certain degree of remission. The corrected C-peptide excretion correlated with the age with of patients indicating a more severe beta-cell damage in the younger patients.

The influence of the aging of the erythrocyte on the insulin binding was studied. It was found that reticulocytes have higher binding capacity for insulin than mature erythrocytes. In patients with normal erythropoiesis the age-related factors constituted 35% and the methodological factors 5% of the variation, while the remaining 60% were independent of these factors. In patients with haematopoetic disease with active erythropoiesis the insulin binding to erythrocytes and their puruvate kinase activity had nearly the same half-life while the fall in the reticulocyte count was much more rapid.

In the children with diabetes mellitus type I as well as in a child with superimposed Addison's disease the insulin binding to erythrocytes was decreased but was restored to normal in the remission phase and after treatment.

The insulin sensitivity was studied using an insulin infusion test. It was demonstrated that the children with more disturbed metabolism were insulin resistant. There was a relationship between the blood glucose decrement and the fasting blood glucose level, the glucose assimilation rate and the blood glucose level at 60 minutes and at 120 minutes during the oral glucose tolerance test. When entering remission phase the retested children had a normal sensitivity. The findings indicate that insulin resistance is not an abnormality associated with diabetes mellitus per se.

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URINARY C-PEPTIDE EXCRETION AT ONSET OF
INSULIN DEPENDENT DIABETES MELLITUS IN CHILDREN

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Abstract

The 24-hour urinary excretion of C-peptide and the plasma C-peptide concentration were measured at the onset of insulin dependent diabetes mellitus (IDDM) in children. The excretion of C-peptide was twice that found in normal control subjects, whereas the plasma C-peptide values were markedly lower, indicating increased urinary leakage of C-peptide in this phase of the disease. There was a significant correlation between the C-peptide excretion and the plasma C-peptide concentrations in both the control children and in those with IDDM. In the diabetic children under seven years of age the plasma C-peptide levels were mostly undetectable and the mean value of C-peptide excretion was also lower than in the older children. The urinary C-peptide excretion correlated with the duration of the remission period and with indices for short term metabolic control. The estimation of 24-hour urinary excretion of C-peptide may be used as an index for short term prognosis.

INTRODUCTION

Several reports now support the view that most patients have residual beta-cell function at the onset of IDDM. The remaining capacity for insulin secretion might be clinically important for at least the short-term prognosis. This conclusion is based on single plasma C-peptide measurement (1-4). In order to get a better idea of the patient's endogenous insulin production it might be advantageous to measure 24-hour urinary C-peptide as compared to a single plasma C-peptide sample (5-7). Furthermore, the problems inherent in the plasma C-peptide and the insulin assays, like the presence of endogenous proinsulin, exogenous insulin or insulin antibodies, do not apply to urinary C-peptide (8). To our knowledge there are no studies on the 24-hour C-peptide excretion in children during the initial hospitalization for diabetes mellitus.

The aims of this study were: to determine the 24-hour C-peptide excretion at the onset of type I diabetes mellitus and 2-4 weeks later during a phase of falling demand for exogenous insulin; to relate the C-peptide excretion to the length of the initial remission and to short-term metabolic control; to study the influence of age on the degree of the beta-cell dysfunction. For comparison urinary and plasma C-peptide were determined in normal children.

Material

Patients: 19 children, 11 boys and 8 girls, with IDDM were studied. Pertinent clinical data are given in table I. All the patients required continued insulin therapy. None were acidotic - i.e., with a standard CO_2 value less than 20 mmol/L. 14 patients had ketonuria - i.e., Ketostix[®] 1+ to 3+. Samples for determination of plasma C-peptide were taken on admission to hospital before insulin therapy was instituted. Urine was carefully collected for 24 h within 6 days of treatment ($m=3.4$ days) and 12-24 days later ($m=17.7$ days) during the initial hospitalization period. The urinary glucose values given in Table 1 are the mean of the urinary glucose collected the day before and after the day for C-peptide collection. The remission phase was defined as the period in which more than 95% of repeated test (Clinitest[®]) performed at home on about three occasions daily did not show any glucosuria and an insulin requirement of less than 0.4 U/kg body weight per 24 h.

Controls: 19 healthy children (16 boys and 3 girls, 9-15 years of age) with no first degree relative with known diabetes volunteered. They were within ± 2 S.D. of ideal weight (9). Blood samples for C-peptide concentration were obtained after an overnight fast, the morning after completing the 24 h urine collection. No effort was made to regulate diet or physical activity during the urine collection. Informed consent was obtained from each of the children and their parents.

Methods

Urine was collected for 24 h in bottles which were prepared with a buffer solution (10% bovine serum albumin in 0.05 M phosphate buffer, pH 7.5, 0.1 g sodium azide and 0.2 g merthiolate). Samples were kept at 5°C during collection. The assay of C-peptide in urine was done as described by Heding (10) with antiserum M 1230. The urine sample was diluted in 6% NaFAM and assayed in three dilutions (1:20, 1:50 and 1:100) before they were determined and the results were linear across the range of dilutions in the assay. In view of the different ages of the children, the values for C-peptide

Table I

Clinical and laboratory data

Patients	Age years	Body surface m ²	Blood glucose ¹ mmol/L	Duration of ketonuria days	Urinary glucose		Urinary glucose	
					Period I ² mmol/24 h	Period II ² mmol/24 h	Period I ² mmol/24 h	Period II ² mmol/24 h
1	3	0.63	12.4	0	243	87		
2	4	0.64	18.5	1	268	-		
3	5	0.72	16.5	1	177	-		
4	5	0.86	22.0	1	64	3		
5	6	0.80	10.7	1	0	4		
6	8	0.97	12.7	1	33	0		
7	8	1.07	10.1	1	75	-		
8	8	1.08	16.6	1	48	-		
9	9	1.02	10.2	1	111	115		
10	9	1.00	15.1	1	271	16		
11	9	1.13	18.5	1	155	77		
12	10	1.13	23.8	2	408	78		
13	11	1.35	18.9	1	288	-		
14	11	1.38	22.0	0	270	0		
15	13	1.45	22.9	0	0	-		
16	13	1.53	28.0	1	165	0		
17	14	1.64	25.7	2	242	11		
18	15	1.26	22.9	0	400	-		
19	15	1.82	17.6	0	82	70		

¹ On admission to hospital² See text

excretion were adjusted to the body surface and are thus given as nmol/24 h/m². Plasma C-peptide was measured according to Heding with antiserum M 1230 (10-11). Plasma and urine were stored at -20°C until analyzed within three weeks. The detection limit of the C-peptide assay for both urine and blood was 0.05 nmol/L. The within and between assay coefficients of variation for urinary C-peptide determinations were 4.3% and 9.6%, respectively.

HbA_{1c} was analyzed as described by Jeppsson et al (12).

Statistical methods

The results are given as mean $\pm$ standard error of mean. Student's t-test, paired Student's t-test and linear regression analysis were used for statistical analysis.

Results

Eighteen of the 19 diabetic children had detectable C-peptide in urine on admission to hospital. In 12 of them C-peptide was also found in plasma. The mean 24 h urinary excretion of C-peptide was 7.11 ± 1.22 (SEM) nmol/24 h/m² which is higher than in normal children (3.66 ± 0.38 nmol/24 h/m², $p < 0.05$). The C-peptide in plasma of the diabetic children was lower (0.14 ± 0.03 nmol/L) than in the controls (0.32 ± 0.02 nmol/L, $p < 0.01$, Table II). Positive correlations were found between the plasma concentration and urinary excretion of C-peptide both in the normal individuals ($r = 0.60$, $p < 0.01$) and in the diabetic children ($r = 0.68$, $p < 0.01$). No correlation was found between urinary C-peptide excretion and HbA_{1c} measured on admission to hospital ($r = -0.08$).

The excretion of C-peptide at 12-24 days after hospitalization was in the same range as initially ($m = 7.41 \pm 2.13$ nmol/24h/m²) (Table II). On this occasion insulin requirement had decreased from 0.51 ± 0.05 to 0.37 ± 0.05 units/kg body weight ($p < 0.01$), and the total urinary glucose secretion had fallen from 174 ± 29 mmol/24 h to 38 ± 12 mmol/24 h ($p < 0.01$). The values for 24 h urinary C-peptide/m² determined on the two occasions correlated well ($r = 0.78$, $p < 0.01$).

Table II

Plasma and urinary C-peptide, duration of the remission phase and the insulin dosage after 12 months

Patients	Plasma C-peptide ¹ nmol/L	Urinary C-peptide Period 12 nmol/m ² /24 h	Urinary C-peptide Period 22 nmol/m ² /24 h	Duration of remission months	Insulin dosage after 12 months units/kg body weight
1	0	4.13	4.29	0	0.82
2	0	4.69	-	0	1.00
3	0	4.17	-	4	0.80
4	0.09	1.02	1.40	0	0.85
5	0	3.05	2.38	1	0.58
6	0.12	2.68	2.85	15	0.31
7	0.06	5.05	-	7	0.80
8	0.40	18.89	-	2	0.66
9	0.23	6.86	8.72	4	0.80
10	0.25	6.25	5.70	6	0.55
11	0	0	3.50	2	0.47
12	0	5.22	12.39	1	0.47
13	0	9.56	-	6	0.50
14	0.30	4.08	2.17	6	0.98
15	0.32	11.03	-	42	0.21
16	0.23	12.81	8.49	30	0.09
17	0.10	14.29	8.77	21	0.17
18	0.05	4.44	-	0	0.65
19	0.40	16.92	28.24	21	0.24

³m₊SEM

0.14±0.03

m₋SEM

0.32±0.02

7.41±2.13

¹ On admission to hospitalNormals
n = 19

3.66±0.38

² See text³ For statistical analysis the value of 0.025 was used when the level was undetectable

There was a positive correlation between 24 h urinary C-peptide (nmol/m^2) and age ($r=0.53$, $p<0.05$). All children under the age of 7 had less than $5 \text{ nmol}/24\text{h}/\text{m}^2$ C-peptide excretion in urine, but only 4 out of 14 in the older group. The two age groups had markedly different mean values for urinary C-peptide excretion ($m=3.41\pm0.65 \text{ nmol}/24\text{h}/\text{m}^2$ and $m=8.43\pm1.50 \text{ nmol}/24\text{h}/\text{m}^2$, respectively, $p<0.01$) and plasma C-peptide ($m=0.04\pm0.01 \text{ nmol}/\text{L}$ and $0.18\pm0.04 \text{ nmol}/\text{L}$, respectively, $p<0.01$) (Table III).

The urinary excretion of C-peptide correlated to the duration of the remission period as well as to the insulin requirement after one year of disease ($r=0.53$ and $r=-0.51$, $p<0.05$ respectively).

A similar correlation was found between plasma C-peptide on admission to hospital and the duration of the remission period ($r=0.51$, $p<0.05$). The younger children mentioned above had a considerably shorter remission period compared to the older ones ($m=1.0\pm0.8$ and 11.6 ± 3.4 months respectively, $p<0.01$) and their insulin demand per kilo body weight after one year was significantly higher ($m=0.81\pm0.07$ units and 0.49 ± 0.07 units, respectively, $p<0.05$) (Table III).

HbA_{1c} after six months of treatment correlated with urinary C-peptide excretion on admission ($r=-0.47$, $p<0.05$). No such correlation was found after 12 months of treatment ($r=-0.15$).

Discussion

In the present group of children with IDDM with mild metabolic derangement the 24-hour urinary C-peptide excretion both at onset of the disease and two weeks later was twice as high as that of normal children. Their maximally stimulated plasma C-peptide values were only one third of that of fasting C-peptide in normals. This paradoxical finding indicates that the urinary leakage of C-peptide is higher in diabetic children than in normals.

Earlier reports by Meistas et al (13) and Rappaport et al (14) have proposed that the urinary excretion of C-peptide reflects the

Table III

Mean $\pm$ SEM of the plasma and the urinary C-peptide, duration of the remission phase and the insulin dosage. Comparison between children aged 3-7 and 8-15

Age year	3-7	8-15	p
Urinary C-peptide nmol/m ² /24 h	3.41 $\pm$ 0.65	8.43 $\pm$ 1.50	<0.01
Plasma C-peptide nmol/L	0.04 $\pm$ 0.01	0.18 $\pm$ 0.04	<0.01
Duration of remission phase months	1.0 $\pm$ 0.08	11.6 $\pm$ 3.4	<0.01
Insulin dosage at 12 months duration units/kg body weight	0.81 $\pm$ 0.07	0.49 $\pm$ 0.07	<0.05

pancreatic C-peptide secretion closely over a wide physiologic range of plasma C-peptide concentration. However, Kolterman et al (15) demonstrated recently that the C-peptide clearance was elevated in NIDDM implying that these patients had a renal leak of C-peptide. In the report of Rappaport et al (14) the patients with IDDM lasting less than 1 year had an average excretion of 34 ug C-peptide whereas this figure was 39 ug in the control group. During this period the insulin and C-peptide pancreatic secretion most likely was considerably less than in the normals. Their data as well as ours thus indicate an increased leakage of C-peptide in the patients with IDDM at least in the early phase of the disease.

The positive correlation between the plasma C-peptide level and the urinary C-peptide secretion indicates that it is not the renal leakage as such which regulates the plasma C-peptide level. The close correlation indicates further that the degree of renal leakage was relatively similar in all children. These facts taken together suggest that the urinary excretion of C-peptide reflects the pancreatic secretion.

The urinary C-peptide excretion after 12-24 days was in the same range as initially and the two values correlated well. This might be interpreted as partial recovery of the beta-cells as at this time the blood glucose stimulus for endogenous insulin secretion ought to have been less intense as judged by the lower urinary glucose levels compared with the values in the initial phase. Ludvigsson & Heding (1) made the same conclusion on the basis of their findings that one month after the onset of the disease, fasting C-peptide values were about the same as those noted under maximal beta-cell stimulation on admission.

We found correlations between urinary C-peptide excretion at the onset of the disease and the duration of the remission period, HbA_{1c} six months later and the insulin requirement after 12 months of treatment. This indicates that a remaining capacity for insulin secretion is important for short-term prognosis. Our findings are in agreement with other retrospective studies in IDDM suggesting that high residual C-peptide secretion is correlated to a

longer remission period (16-17), lower requirements for exogenous insulin (14) and a better metabolic control (18-20). The absence of any correlation between $\text{HbA}_{1\text{C}}$ and the plasma or urinary C-peptide on admission indicates that $\text{HbA}_{1\text{C}}$ does not reflect the functional status of the pancreas in the initial phase of the disease.

In children under seven years of age the plasma C-peptide levels were mostly undetectable and the average value for urinary C-peptide excretion was much lower than in older children. Possibly, as a consequence of this more severe beta-cell damage the younger children had a shorter remission period and a higher late insulin requirement. Our findings are in accordance with those of Ludvigsson et al (18) who reported a lesser degree of remaining beta-cell function in the youngest age group as measured by fasting plasma C-peptide beyond the post-initial remission period. Crossley et al (21) proposed the hypothesis that the same diabetogenic insult has a greater impact in a younger child because of the smaller mass of beta-cells at a younger age. Earlier reports of different sex ratio (22-25) the lack of seasonal variation (23-24) and a higher prevalence of diabetes in the family in the youngest children (22,25) might on the other hand indicate a different etiology of the disease in this age group.

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Reticulocytes and Insulin Binding to Erythrocytes¹

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ABSTRACT. The relationship between erythrocyte insulin receptors and the reticulocytes were studied in a group with a varying degree of reticulocytosis. The maximal ¹²⁵I-insulin binding to erythrocytes varied between 6% and 37% and it showed a highly significant positive correlation with the reticulocyte count ($r=0.85$). The highest binding was found in a patient with hereditary spherocytosis and very active hematopoiesis (312×10^9 reticulocytes per litre). The results suggest that insulin receptors are predominantly localized to the young erythrocytes, and that further studies are needed to characterize the dependence of the receptor on erythrocyte maturation.

Key words: insulin receptors, erythrocytes, reticulocytes, hereditary spherocytosis.

Determination of insulin receptors in clinical studies has been restricted by the fact that the available methods require either open biopsy (adipocyte receptors) (1, 2, 3, 4) or collection of excessive amounts of blood (monocyte receptors) (4, 5, 6, 7). The method for measuring insulin binding to erythrocytes recently described by Gambhir et al. (8) makes it possible to determine insulin receptors more easily even as a routine method in clinical medicine.

However, Thomopoulos et al. (9) described that in rabbit the binding occurred only to the youngest erythrocytes and that the erythrocytes lost the ability to bind insulin during the process of maturation. In this preliminary investigation we have studied a group of patients with a varying degree of reticulocytosis to evaluate a possible correlation between the proportion of young cells as reflected in the reticulocyte count and the insulin binding to erythrocytes.

MATERIAL AND METHOD

Patients. 12 patients were included in this study. Some pertinent data are given in Tables I and II.

Erythrocyte preparation. 10 ml blood was collected after an overnight fast in heparinized tubes and analysed within 24 hours. After centrifugation (10 min, $400 \times g$,

20°C) the plasma was aspirated. The erythrocytes (RBC) were separated from other blood cells by a modification of the method of Böyum as reported by Gambhir et al. (8). The red blood cell pellet was resuspended in a buffer containing hepes 50 mmol/l, tris 50 mmol/l, $MgCl_2$ 10 mmol/l, ethylenediaminetetra-acetic acid 2 mmol/l, dextrose 10 mmol/l, $CaCl_2$ 10 mmol/l, NaCl 50 mmol/l, KCl 5 mmol/l and 0.1% BSA (pH adjusted to 8.0 at 23–25°C). The final cell concentration was $3.5\text{--}5.1 \times 10^{12}$ cells per litre. The reticulocytes were counted as described by Atwaker (10).

Iodination of insulin. Monocomponent porcine insulin (Novo AS, Copenhagen, Denmark) was iodinated by the lactoperoxidase method according to Thorell and Johansson (11) to a specific activity of 190–215 μCi per μg .

Binding studies. The incubation of RBC and the separation of insulin bound to RBC from free insulin was performed as described by Gambhir et al. (8) with the following modification. Each assay tube contained 400 μl RBC, 25 μl ¹²⁵I-insulin and a varying concentration of unlabelled insulin, in a total volume of 525 μl . After incubation, 100 μl of the suspension was aliquoted into prechilled microfuge tubes containing 100 μl of buffer and 100 μl of dibutylphthalate (Merck-Schuchardt). The tubes were centrifuged in a Beckman microfuge B at 4°C for 2.5 min. The tubes were cut above the pellet leaving about 1/5–1/10 of the total dibutylphthalate volume in the bottom of the tube. The radioactivity of the pellet was measured in a well type gamma counter (Nuclear Enterprises NE 1600). The correction for unspecific insulin binding was based on the activity in the pellet in tubes containing 1×10^4 ng/ml of unlabelled insulin. The unspecific binding was usually less than 5 per cent.

Binding data were calculated from plots of the specific bound radioactivity versus the bound/free (B/F) ratio according to Scatchard (12).

RESULTS

The maximal specific binding (in tubes containing no unlabelled insulin) varied between 6.1–37.1%. The highest binding was found in a patient with hereditary spherocytosis and a high proportion of hereditary spherocytosis and a high proportion of reticulocytes (312×10^9 per litre = 7.8%) (Fig. 1). Within the whole group there was a highly signifi-

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Table I. *Clinical and metabolic data*

	Age	Sex	Diagnosis	Therapy	B-Glucose (mmol/l)	P-Insulin (mIU/l)	Body- weight (kg)
1	69	F	Hemolytic anemia	Cortisone-Propanolol	4.4	3	65
2	38	M	Healthy ^a	—	5.5	6	84
3	23	M	Hereditary spherocytosis	—	4.0	<3	59
4	71	M	Cardiac failure	Hydrochlorothiazid	4.5	4	68
5	34	M	Liver cirrhosis	—	5.1	68	64
6	37	M	Healthy ^a	—	4.8	10	80
7	28	F	Healthy ^a	—	4.5	<3	63
8	10	F	Healthy ^a	—	4.9	<3	30
9	10	F	Healthy ^a	—	5.0	5	29
10	0 ^b	F	Hyperviscosity syndrome	—	4.6	22	2.7
11	0 ^b	F	Congenital cardiac abnormalities, Mb. Down, Hyperviscosity syndrome	—	3.4	16	3.8
12	69	F	Sideropenic anemia	—	5.0	6	62
Ref. value					3.3–5.6	<20	

^a Cases studied without abnormality been revealed.^b Newborn.

cant positive correlation between the binding and the reticulocyte count ($r=0.85$, $p<0.001$) (Fig. 2). No correlation was found to other cellular components.

The binding capacity, calculated from the Scatchard plot, was approximately 50 binding sites per erythrocyte (range 10–120). The reticulocyte constituted 0.7–7.8% of the erythrocyte population. If the receptor sites instead were assumed to be localized on the reticulocytes only (range 34–312×10⁶ per litre), each of them would contain about 3000 sites per cell (range 2500–5000). In all

cases the Scatchard plot was courvilinear. The slope of the high avidity component was approximately identical in all cases. Fig. 3 A and 3 B illustrate the findings in patients with a low and a high reticulocyte count, respectively.

DISCUSSION

These results show that the binding of insulin to erythrocytes is related to the number of reticulocytes. The clinical conditions and other parameters

Table II. *Hematological data*

Leucocytes											
Hb (g/l)	Erythro- cytes (10 ¹² /l)	Total (10 ⁹ /l)	Differential count					Plate- lets (10 ⁹ /l)	Hemato- crit (%)	Reticulo- cytes (10 ⁹ /l)	
			Neutro- phils (%)	Eosino- phils (%)	Baso- phils (%)	Lymfo- cytes (%)	Mono- cytes (%)				
1	116	3.9	5.1	66	0	0	31	3	295	34	156
2	175	4.2	6.5	63	2	0	34	1	160	48	67
3	155	4.0	7.2	68	0	0	30	2	233	40	312
4	183	5.1	5.2	48	5	1	44	2	160	55	82
5	110	3.9	7.0	—	—	—	—	—	275	32	141
6	168	4.6	5.4	60	2	0	33	5	253	45	42
7	138	3.9	4.3	46	4	0	47	3	350	42	76
8	138	4.6	5.0	36	0	1	57	6	335	41	34
9	129	4.9	4.2	44	1	0	54	1	185	40	35
10	260	3.4	—	—	—	—	—	—	250	81	184
11	238	3.3	18.4	70	0	0	27	3	55	80	61
12	50	4.1	8.2	73	0	1	22	5	233	—	182
Ref. value											
value	115–166	3.7–5.4	4–10	50–70	1–4	0–1	20–50	5–8	125–340	37–49	5–70

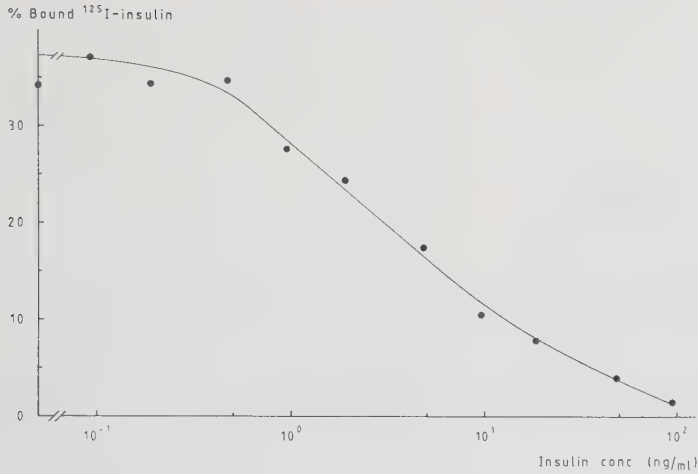


Fig. 1. The per cent of ^{125}I -insulin bound by RBC at various insulin concentrations in a patient with hereditary spherocytosis and a high reticulocyte count (312×10^9 per litre).

such as age and weight of the patients investigated varied markedly, since they were selected primarily because of their reticulocyte count. The rather close relationship found between the insulin binding and the degree of reticulocytosis despite their heterogeneity indicates that the number of reticulocytes is an important denominator for the insulin binding. This relationship has recently been studied by others with divergent results. Eng et al. (13) reported a close correlation but Kappy et al. (14) were unable to find any relationship. The lack of

correlation in the study of Kappy et al. may depend on a small variation in the number of reticulocytes which hid the correlation among other factors. This is accentuated by the rather poor precision of available methods for estimating the number of reticulocytes.

HbA _{1c} (%)	S-Vitamin B ₁₂ (pmol/l)	S-Iron ($\mu\text{mol/l}$)	S-TIBC ($\mu\text{mol/l}$)
7.0	270	16	61
6.9	380	27	97
6.4	200	18	73
6.6	260	17	75
—	—	—	—
7.1	353	22	67
7.3	230	17	66
8.3	450	12	81
7.2	313	13	79
—	—	—	—
—	230	4	91
5.8–8.2	110–650	10–35	45–72

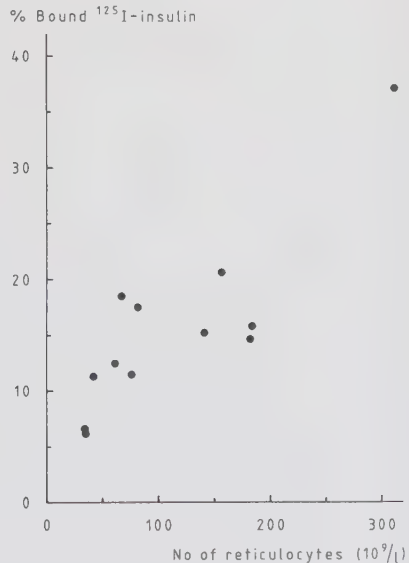


Fig. 2. ^{125}I -insulin binding (%) to erythrocyte receptors as a function of reticulocyte count in blood. Corr. coeff.: 0.85 ($p < 0.001$).

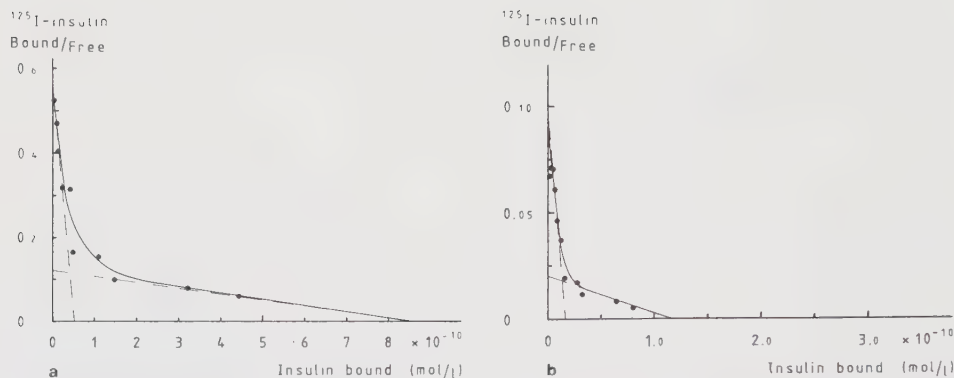


Fig. 3. Scatchard plot. Bound-to-free ^{125}I -insulin versus total insulin bound specifically. (A) Patient with high re-

ticulocyte count (312×10^9 per litre). (B) Patient with low reticulocyte count (34×10^9 per litre).

The studies do not reveal whether the correlation between the insulin binding and the number of reticulocytes is an effect of a selective binding to the young erythrocytes only, or if it is caused by some event related to the turnover rate of the erythrocytes. Reported estimates of the binding sites per erythrocyte have given different results. This study showed 10–120 sites per cell, which agrees with studies of Kappy, Insel and Wachslicht-Rodbard (14, 15, 16). Gambhir et al. (8) found as many as 2000 sites per cell. The lower number is probably more correct since it includes only the high avidity binding sites. The biological significance of the low avidity-high capacity binding is questionable because the binding is minimal at physiological concentrations of insulin. The number of active sites found per cell is much lower than in other known target cells for insulin effects. This suggests that only a subpopulation of the erythrocytes has the ability to bind insulin. Calculation of the number of receptor sites per reticulocyte rather than to all erythrocytes gives 2500–5000 sites per cell. This is in the same order as the number of receptor sites calculated from monocytes (7, 17) but still lower than in adipocytes and hepatocytes (3, 4).

The highest insulin binding was found in a patient with a hemolytic condition because of a hereditary spherocytosis. Other conditions with a high rate of reticulocyte production and an increased insulin binding involves experimentally induced hemolysis in rabbits (9) and the remission phase by vitamin B_{12} treatment of pernicious anemia (13). However, in the later condition the binding remained high even

when the number of reticulocytes was normalized. This suggests that the binding is confined not only to the youngest erythrocytes but occurs beyond the reticulocyte stage.

This study indicates that measurement of the insulin binding to the total erythrocyte population does not accurately reflect the concentration of receptors since the results are influenced by the proportion of receptor containing cells as well. Further studies are needed to characterize the dependence of the receptors on erythrocyte maturation.

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THE INFLUENCE OF ERYTHROCYTE AGE ON ESTIMATIONS OF
ERYTHROCYTE INSULIN BINDING IN HEALTHY CHILDREN AND
ADULTS AND IN CONDITIONS WITH INCREASED ERYTHROPOIESIS

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Abstract

Insulin binding to erythrocytes was shown to lack any relationship to age-related factors of the erythrocyte such as the pyruvate kinase activity and the reticulocyte count in individuals with a normal erythropoiesis. After density separation of the erythrocytes in both normal adults and children and in individuals with increased erythropoiesis, a correlation to age-factors was evident (pyruvate kinase activity/insulin binding $r=0.59$, $p<0.01$, reticulocyte count/insulin binding $r=0.44$, $p<0.01$). An estimate of the components of variation in the binding showed that the unexplained variance is 60%. In the density separated samples the insulin binding correlated closer to the pyruvate kinase activity than to the number of reticulocytes. During treatment of haematopoietic diseases in the four patients with increased erythropoiesis, the disappearance rate for the reticulocyte count was much faster ($T_{1/2} = 9$ days) than that of the insulin binding to erythrocytes ($T_{1/2} = 37$ days) and that of pyruvate kinase activity ($T_{1/2} = 36$ days). In one patient studied after splenectomy, the decrease in insulin binding paralleled the disappearance curve for ^{51}Cr -tagged erythrocytes. These results suggest that insulin binds to erythrocytes, not only during the reticulocyte stage, but throughout their life span, though the binding, which is very closely related to their pyruvate kinase activity, steadily declines as the erythrocytes age.

The human erythrocyte has been used as a cellular model for assessing the insulin receptor in general. The erythrocyte has specific surface insulin receptors with characteristics similar to those of the receptor found in classical target tissues for insulin action.^{1,2} As was first shown by Thomopoulos et al.,³ one problem associated with the erythrocyte model is the heterogeneity of the insulin binding depending on the age of the erythrocytes. The reticulocytes have higher binding capacity for insulin than do mature erythrocytes,⁴⁻⁶ and there is a marked increase of erythrocyte insulin receptors in diseases in which very young erythrocytes are abnormally frequent.⁶ Kosmakos et al.,⁴ demonstrated that insulin binding was closer correlated with the number of reticulocytes than with the number of erythrocytes, and Eng et al.,⁵ found a high insulin binding in studies of patients with haemolytic states. It has therefore been questioned⁷ whether the measurement of erythrocyte insulin receptors reflects changes in the insulin binding in general, or whether it is primarily a function of erythrocyte age distribution. However, it has not yet been determined to what extent the age factor affects the measurement of insulin receptors in individuals with normal erythrocyte production and degradation.

In the present study we have investigated the binding of insulin as a function of erythrocyte age in healthy children and adults, and in four patients with altered erythropoiesis.

Materials and methods

Healthy subjects: Twentythree individuals were studied, 9 children (11-16 years old) and 14 adults (22-53 years old). They were all healthy, with normal body weight and haematologic status.

Patients: Four patients with increased erythropoiesis were studied. Two women, 72 and 68 years old, with untreated pernicious anaemia were followed during the remission phase after cyanocobolamin treatment, the study of the 72 year-old being started 2 weeks after the first cyanocobolamin injection, and that

of the 68 year-old 4 days after the first injection. A 54 year old woman, with drug induced haemolysis due to treatment with dapson (Avlosulfan[®]), was followed during remission. A 23 year old man, with haemolysis due to hereditary spherocytosis, was studied after undergoing splenectomy.

Red cell sample collection, separation and receptor assay:

Blood was sampled after an overnight fast.

Insulin binding to erythrocytes was measured as described by Gambhir et al.,⁸ with certain modifications of the receptor assay.⁶ The specific cell-bound fractions were adjusted to a standard erythrocyte concentration of 4.4×10^{12} cells/L. Binding data were expressed as the specific binding of the sample containing no unlabelled insulin (%B/T). The binding values, estimated from duplicate determinations, had an intra-assay coefficient of variation of 6.3% (SD = 0.7), and the inter-assay coefficient of variation of repeated measurements of the same samples on two consecutive days was 8.0% (SD = 0.8).

In a separate study, comprising 9 healthy adults and children, the erythrocytes were density fractionated with a method modified from that described by Danon and Marikovsky.⁹ Ten ml blood was centrifuged at 20°C, 10 min, 400 x g and plasma was aspirated. The red blood cell was resuspended in an equal volume of 50 mM Hepes buffer,⁸ pH 8.0. The suspension was superimposed on a phthalate solution with specific gravity (SG) 1.096 (a mixture of methylphthalate, SG 1.189, Eastman-Kodak & Co. and dibutylphthalate, SG 1.0416, Eastman-Kodak & Co. SG being checked with a hydrometer) and centrifuged for 1 h at 15,000 r.p.m. at 30°C in a Sorvall Superspeed RC2-B with the SS-34 rotor. The top buffer layer was discarded, and the low and high density fractions of the erythrocytes were transferred into separate tubes. The fractions were resuspended in NaCl and centrifuged in 20°C, 5 min, 400 x g and the NaCl was aspirated. The red blood cell pellet was resuspended in an equal volume of Hepes buffer. Blood was drawn to determine erythrocytes, reticulocytes and pyruvate kinase

Table 1

Reticulocyte count, pyruvate kinase activity and specific ^{125}I -insulin binding to erythrocytes (mean $\pm$ SEM) after density fractionation in normal adults and children

	Reticulocytes $\times 10^9/\text{L}$	Pyruvate kinase U/L erythrocytes	Specific Bound ^{125}I -insulin %
<u>Adults</u>			
Low density fraction (L)	32.3 $\pm$ 7.0	3.7 $\pm$ 0.2	21.8 $\pm$ 0.8
High density fraction (H)	8.5 $\pm$ 1.0	2.5 $\pm$ 0.1	15.5 $\pm$ 1.1
p (difference between L and H)	<0.01	<0.01	<0.01
Ratio $\frac{\text{L}}{\text{H}}$	3.8	1.5	1.4
<u>Children</u>			
Low density fraction (L)	21.1 $\pm$ 5.4	2.9 $\pm$ 0.2	21.8 $\pm$ 1.2
High density fraction (H)	4.6 $\pm$ 1.1	1.9 $\pm$ 0.2	13.7 $\pm$ 0.6
p (difference between L and H)	<0.01	<0.01	<0.01
Ratio $\frac{\text{L}}{\text{H}}$	4.6	1.5	1.6

activity. The insulin binding was measured as for the unseparated samples, but for incubation overnight.

Iodination of insulin: Monocomponent porcine insulin (Novo AS, Copenhagen, Denmark) was iodinated by the lacto-peroxidase method according to Thorell and Johansson¹⁰ to a specific activity of 209 uCi/ug (range 191-222 uCi/ug). The ¹²⁵I-insulin showed binding characteristics of A 14 mono-iodinated insulin.¹¹

Other assays

Erythrocytes were counted in duplicate with a Linson 431 celloscope, and once in a Linson 401 celloscope (Linson Instrument AB, Sweden). The mean of these three values was used.

Reticulocytes were counted on two separate occasions as described by Atwaker & Erslev¹² and the mean was used.

The pyruvate kinase activity was measured as described by Amador et al.¹³ The results are given in units per L of packed erythrocytes.

Erythrocyte survivaltime was measured according to the standard method with ⁵¹Cr.¹⁴

Statistical methods

The results are given as mean $\pm$ standard error of mean. For comparison between low and high density fractions, the paired Student's t-test was used. Regression analysis was calculated with standard procedure.

Results

There was no correlation between insulin binding and two age-dependent factors of the erythrocyte, the pyruvate kinase activity ($r = -0.39$, $p > 0.05$) and the reticulocyte count ($r = -0.15$, $p > 0.05$).

Table 2

Longitudinal studies of specific ^{125}I -insulin binding, pyruvate kinase activity and reticulocyte count in patients with increased erythropoiesis during a period of normalization

Patient number	Day	Specific Bound ^{125}I -insulin %	Reticulocytes $10^9/\text{L}$	Pyruvate kinase U/L erythrocytes
1	0	29.2	56	4.1
	7	29.6	30	3.0
	17	17.1	17.5	2.2
	31	14.7	11.5	2.2
	52	8.4	6.5	2.4
	82	8.2	4.5	1.9
2	0	21.4	154.5	-
	8	19.9	51.5	-
	15	15.3	23.5	-
	22	14.9	27	-
	36	14.0	10.5	-
	57	11.4	8.5	-
	80	6.0	6.5	-
3	0	27.7	77	6.5
	6	23.6	23.5	3.6
	13	20.8	18.5	3.2
	27	19.3	8.5	3.4
	44	9.4	10	2.9
	75	8.6	20.5	2.8
4	0	37.1	312	-
	6	33.7	161	-
	13	27.7	49	-
	20	22.9	16	-

After fractional centrifugation of the samples in a density medium, a high proportion of reticulocytes and erythrocytes rich in pyruvate kinase were recovered in the portion above the separating medium. They exhibited a higher insulin binding than the cells below the medium (Table 1). The ratio between the binding in the low and the high density fractions (upper/lower) was about 1.5. The same ratio was found for the pyruvate kinase activity, whereas the ratio for the reticulocyte count between the fractions was as high as 3.8-4.6 ($p < 0.01$), indicating that the insulin binding is not determined by the number of reticulocytes only.

After density separation positive correlations appeared between the insulin binding and both the reticulocyte count and the pyruvate kinase activity (Fig. 1A and B). Analysis of the components of the total variance of the binding for all fractionated samples showed that the methodological variations constituted 5%, the age related variation 35%, while the remaining 60% was independent of these factors.

However, the relationship between the binding and the age factors in the two separated samples from each individual (Fig. 1A and B) showed a considerable inter-individual variation. This was most obvious for the relationship between the binding and the reticulocyte count, which was markedly non-parallel among the individuals.

In patients with haematological diseases with hyperactive erythropoiesis, the insulin binding was very high and showed a positive correlation both to the reticulocyte number ($r = 0.69$, $p < 0.01$) and to the pyruvate kinase activity ($r = 0.65$, $p < 0.05$). During the period with decreasing erythropoietic activity, the insulin binding, the number of reticulocytes, and the pyruvate kinase activity fell markedly, though the fall in the reticulocyte count was much more rapid than the decrement of the binding and the pyruvate kinase activity (Table 2). The average half-life of the reticulocyte decrement during the first month was 9 days, while the half-lives for the decrease in insulin binding and

Fig. 1 a

Specific ^{125}I -insulin binding (%) to high density (●) and low density (○) fractionated erythrocytes versus reticulocyte count ($r=0.44$, $p<0.01$)

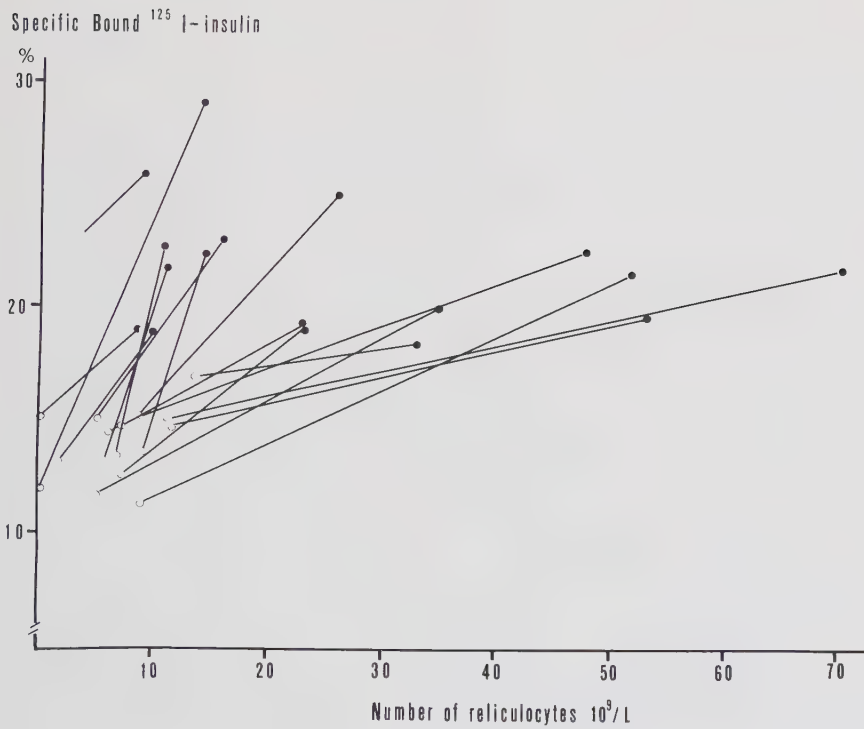
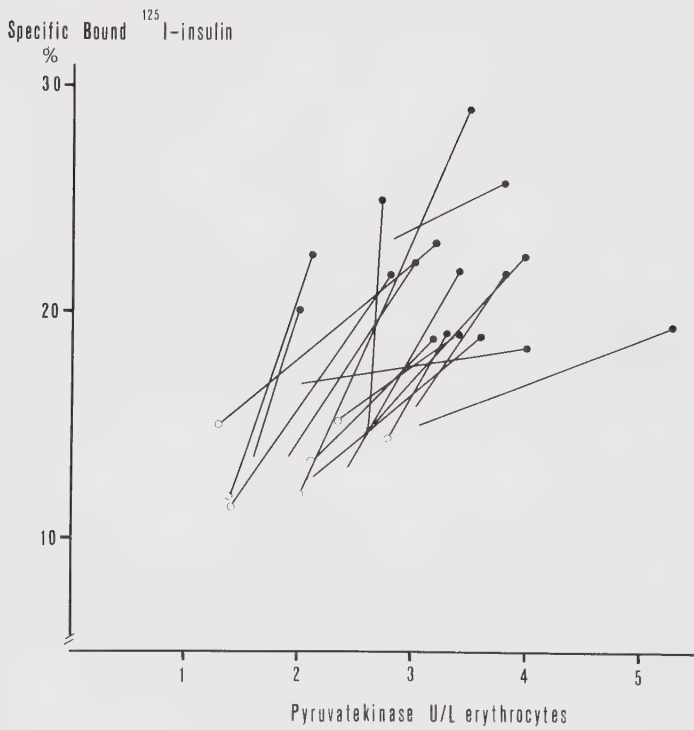


Fig. 1 b

Specific ^{125}I -insulin binding (%) to high density (●) and low density (○) fractionated erythrocytes versus pyruvate kinase activity ($r=0.59$, $p<0.01$)



pyruvate kinase activity were 37 and 36 days respectively. In all patients the number of reticulocytes was normalized long before the insulin binding had reached a steady state level.

The insulin binding and the turn over rate of the erythrocytes were studied during the period following splenectomy in a patient with hereditary spherocytosis (Fig 2). The binding decrease paralleled the disappearance curve of the ^{51}Cr -tagged erythrocytes, both curves having a linear course with half lives of 28 and 29 days respectively. The half-life for the fall of reticulocytes in the same study was 5 days.

Discussion

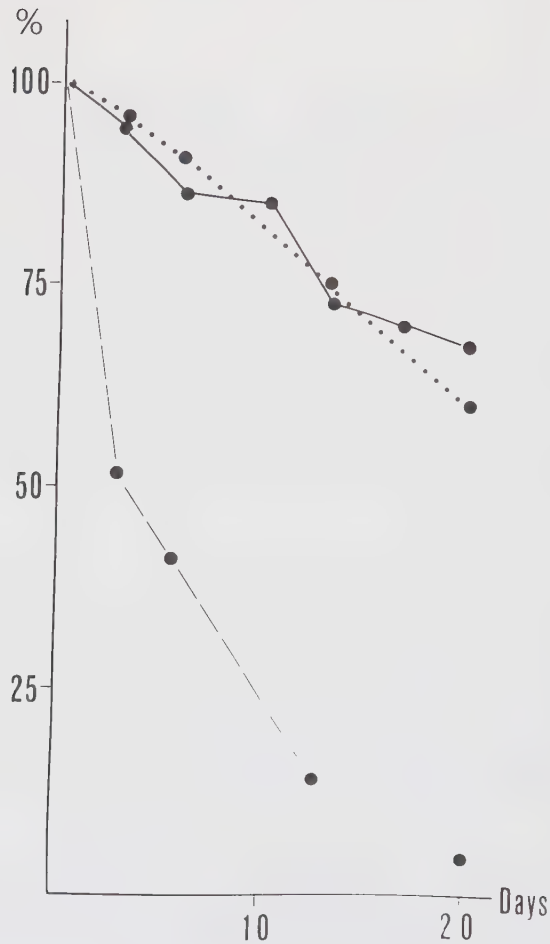
These studies have verified a relationship between the insulin binding to erythrocytes and both the number of reticulocytes^{4,5,7,15,16} and their pyruvate kinase activity, another factor varying with the age of the erythrocytes.^{17,15,18} It was demonstrated in patients with increased erythropoiesis and in normal individuals after density fractionation of the erythrocytes, but we were unable to show any relationship between the insulin binding and these age-related factors in unfractionated samples from individuals with normal erythropoiesis. This is in accordance with the few previous studies in which this was studied.^{19,20}

The discrepancy between the results in fractionated and unfractionated samples is caused by the separation of the erythrocytes into two subpopulations - one containing predominantly young cells with relatively high binding, the other mostly old cells with lower binding. It amplifies the effect of the age factor and causes a correlation which is not apparent in unfractionated samples in which the estimate of the binding represent an average for erythrocytes of all ages.

The analysis of the components of variation in the binding in unfractionated samples demonstrated clearly that factors unrelated to age had a much larger influence than that caused by the age of

Fig. 2

Specific ^{125}I -insulin binding (dotted line), ^{51}Cr -tagged erythrocytes (uninterrupted line) and number of reticulocytes (interrupted line) during the period immediately after splenectomy in the patient with hereditary spherocytosis, expressed in per cent of initial values.



the erythrocytes. It is not until the variation in the age component exceeds the normal range like in haemolytic conditions, that the influence of the age factor will increase to such an extent that it hides the effects of the non-age dependent factors.

In density fractionated samples of erythrocytes, the binding showed a closer relationship to the pyruvate kinase activity than to the reticulocyte count. In patients with haematopoietic diseases studied during a period of normalization after highly increased erythropoiesis, the fall in the reticulocyte count was much more rapid than that of the insulin binding or the pyruvate kinase activity. The in vivo study of Dons et al.,¹⁵ indicates that the insulin receptor concentration decreased continuously as an exponential function of erythrocyte age. The binding of insulin to older erythrocytes as well as to the reticulocytes is also supported by our study of the ⁵¹Cr-labelled erythrocytes in the patient with hereditary spherocytosis investigated after splenectomy. The number of ⁵¹Cr-labelled erythrocytes and the insulin binding decreased in an almost parallel fashion, and they reached a steady state much later than did the number of reticulocytes.

The dependence of the erythrocyte insulin binding on other factors than the age of erythrocytes is supported by comparative studies of the binding to erythrocytes, monocytes and fat cells.^{16,21-25} However, certain differences between the erythrocytes and the other insulin receptors containing cell types may affect their reactions to metabolic influences. The mature erythrocyte is unable to modify its concentration of insulin receptors since protein synthesis, a requirement for up- and down-regulation of receptors is very limited in the absence of a nucleus.²⁶⁻²⁸ The erythrocyte content of insulin receptors is therefore probably established during the period of development. Our results indicate that the receptors of the mature erythrocytes disappear gradually as they age. If the decrease in receptor concentration occurs at a constant rate, the insulin binding to erythrocytes will reflect the conditions prevailing during their early stage of development. The long half-life of the erythrocytes

make them unsuitable indicator of short term changes in insulin receptor contents as indicated by discrepancies between the insulin binding to erythrocytes and monocytes found in studies involving short-term alterations in the metabolic status of the patients.^{23,24,29}

Thus it is likely that chronic metabolic influences affect the average receptor concentration of the erythrocyte population^{21,23,30,31} in the same way as for other insulin receptor containing cells. In individuals with a normal erythropoiesis the influence of the erythrocyte age in receptor content has negligible influence on the estimate of the insulin binding. The results show also that the introduction of a correction factor - not only for the reticulocyte count as suggested by Ward et al.¹⁶ but also for the pyruvate kinase activity as suggested by Camagna et al.¹⁸ - will introduce additional errors.

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Insulin Binding to Erythrocytes in Children with Type I Diabetes Mellitus before, and after Insulin Treatment

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Summary

^{125}I -insulin binding to erythrocytes was studied in 24 children with type I diabetes mellitus before institution of insulin therapy, and in 10 normals. Mean specific ^{125}I -insulin binding in the diabetics was significantly lower than in the controls ($10.2 \pm 0.4\%$ (SEM), as against $11.6 \pm 0.5\%$, $P < 0.05$). In 8 diabetics the study was repeated after 3–6 weeks of therapy, at which time the insulin binding had increased to normal (12.1% , range 10.7–13.6). No correlation was found between insulin binding and blood glucose, plasma insulin or plasma β -hydroxybutyrate. We conclude that insulin binding to the receptors is altered in the early phase of type I diabetes.

Key-Words: *Insulin Receptor — Erythrocytes — Type I Diabetes*

Introduction

The course of events in the development of type I diabetes before it becomes clinical overt and can be diagnosed, are poorly understood. In addition to the β -cell insufficiency, other precipitating factors may be involved (Cahill and McDewitt 1981). The involvement of the insulin receptor in this development is uncertain. The occurrence of auto-antibodies capable of binding to the receptor in juvenile diabetics is a finding which indicates that alterations at the receptor level may play a role (Maron, Elias, de Jongh, Bruinings, van Rood, Shechter and Cohen 1983). Recently we reported the occurrence of decreased insulin sensitivity in certain children with newly diagnosed diabetes mellitus*, characterized by a severe metabolic disturbance. This result also supports that the peripheral effect of insulin may be disturbed in the initial phase of the disease. Available data on insulin binding in children with untreated diabetes type I are very scanty and are limited to a few patients under the age of 15 (Pedersen, Beck-Nielsen, Sorensen and Svegaard 1982).

This report describes insulin binding to erythrocytes in twentyfour newly detected, untreated children with type I diabetes. The relation of insulin binding to other variables

reflecting the degree of insulinopenia and metabolic disturbance was also studied. Some children were re-examined 3–6 weeks later when most of them were in remission.

Materials and Methods

Patients

24 children with type I diabetes (9 girls and 15 boys) were studied. They were referred to the hospital because of newly detected glucosuria, polydipsia or polyuria. Blood samples were collected before the initiation of any therapy. Relevant clinical data are given in Table 1. All the patients required continual insulin therapy. Eight patients were retested after they had been treated with insulin and diet for 3–6 weeks, most of them then being in a remission, i.e. 90% of repeated tests (Clinitest®) performed at home not showing any glucosuria and an insulin requirement less than 0.4 U/kg body weight.

Controls

10 healthy children (2 girls and 8 boys) with no first degree relative with known diabetes volunteered. They were between 11 and 16 years of age and within ± 2 SD of ideal weight (Karlberg, Landström, Lichtenstein, Lindström and Svanberg-Redegren 1976). Their blood was sampled in the morning after an overnight fast. Informed consent to participate in the trial was obtained from both children and parents in both groups and the project was approved by the Medical Ethics Committee, University of Lund.

Receptor assay

10 ml blood was collected in heparinized tubes and analysed within 30 hours. After centrifugation (10 minutes, $400 \times g$, 20°C) the plasma was aspirated. The ^{125}I -insulin binding to erythrocytes (RBC) was determined in duplicates according to the method of Gambhir et al. (Gambhir, Archer and Carter 1977; Gambhir, Archer and Bradley 1978) with the following minor modifications. Each assay tube contained 400 μl RBC, 25 μl ^{125}I -insulin, and a varying concentration of unlabelled insulin in a total volume of 525 μl . After incubation, aliquots of 100 μl of the suspension were added to pre-chilled microfuge tubes containing 100 μl of buffer and 100 μl of dibutylphthalate (Merck-Schuchardt). The tubes were centrifuged in a Beckman microfuge B at 4°C for 2.5 minutes, and then cut above the pellet leaving about 1/5–1/10 of the total dibutylphthalate volume in the bottom of the tube. The radioactivity of the pellet was measured in a well-type gamma counter (Nuclear Enterprises NE 1600). The correction for unspecific binding was based on the activity of the pellet in tubes containing 4760 $\mu\text{g/L}$ of unlabelled insulin. The binding studies were performed with $2.6\text{--}4.1 \times 10^{12}$ erythrocytes/L, but all values were expressed as the binding of 4.4×10^{12} erythrocytes/L. The binding was calculated as the maximal binding at tracer concentration of insulin, although a full standard curve was performed in each case. Binding data were calculated from plots of the specific bound radioactivity versus the bound/free (B/F) ratio according to

* Ivarsson et al.: Unpublished results.

Table 1 Clinical data

Patient	Sex	Age year	Blood glucose mmol/L	Keton- uria	P- β - OHBA mmol/L	Plasma- insulin mU/L	Plasma- C-peptide nmol/L	Insulin binding %
1	F	3	14.1	0	1.54	8	0.14	9.8
2	M	4	38.1	++	1.06	5	0.09	11.7
3	F	4	36.0	0	0.277	< 3	0.18	9.5
4	M	4	28.6	+	1.40	11	0.11	10.2
5	M	5	12.5	++	5.36	< 3	0.09	7.4
6	M	5	4.1	0	0.666	< 3	0.11	9.7
7	M	5	17.3	0	0.114	9	0.33	7.9
8	M	5	18.0	++	3.64	< 3	0.10	12.5
9	F	6	14.8	0	—	< 3	0.07	10.0
10	F	7	17.9	+	1.56	6	0.20	13.2
11	F	7	14.7	+	2.33	< 3	0.09	9.2
12	M	8	5.1	0	0.145	4	0.21	11.4
13	F	9	16.2	+++	3.64	< 3	0.24	8.9
14	M	12	14.3	++	4.82	< 3	—	12.4
15	F	12	16.5	+++	3.61	31	0.39	8.5
16	M	12	9.7	0	0.097	15	0.23	11.9
17	F	12	10.0	0	0.685	7	< 0.03	8.1
18	M	13	22.1	0	0.870	9	0.11	18.0
19	M	13	22.9	0	0.194	16	0.32	12.0
20	F	13	43.9	+++	17.4	10	0.05	8.8
21	M	14	55.5	++	4.84	< 3	0.03	9.4
22	M	14	34.2	+++	9.02	< 3	0.12	8.3
23	M	14	14.4	+++	5.42	7	0.29	8.9
24	M	15	15.0	++	1.56	< 3	0.26	10.9
$\bar{X} \pm \text{SEM}$			20.7 $\pm$ 2.6		3.054 $\pm$ 0.809	7 $\pm$ 1	0.16 $\pm$ 0.02	10.1 $\pm$ 0.4

Scatchard (1949) and affinity profile was calculated as described by De Meyts and Roth (1975).

Other assays

Blood glucose was measured by a hexokinase method (Carroll, Smith and Babson 1970), plasma insulin by radioimmunoassay with ethanol precipitation of antibodies (Thorell and Johansson 1971), plasma-C-peptide according to Heding with the antiserum M 1230 (Heding 1975; Faber, Markussen, Naithani and Binder 1976) and plasma- β -hydroxybutyrate according to Persson (1969). Reticulocytes were counted as described by Atwaker (1972).

Statistical methods

The results are given as mean $\pm$ standard error of mean. The Mann-Whitney-Wilcoxon two sample test, and the Wilcoxon test for paired differences were used for comparison of values between groups and within groups, respectively.

Results

The erythrocyte binding of ^{125}I -insulin at the onset of diabetes mellitus, before insulin therapy had been started, was 10.2 ± 0.4 per cent (range 7.4–13.2) (Table 1). This is significantly lower than the binding of 11.6 ± 0.5 per cent ($P < 0.05$) in normals (range 9.1–14.1).

The affinity of the binding did not differ between the groups K_D in the diabetic group was $7.8 \times 10^8 \text{ M}^{-1}$, compared with $8.0 \times 10^8 \text{ M}^{-1}$ in the normals K_D was $3.8 \times 10^8 \text{ M}^{-1}$ in the diabetics compared with $4.3 \times 10^8 \text{ M}^{-1}$ in the normals.

Table 2 Insulin binding to erythrocytes before and after therapy

Patient number	Insulin binding		Duration of therapy (weeks)
	Before therapy %	After therapy %	
3	9.5	11.4	6
10	13.2	13.3	6
11	9.2	11.6	6
13	8.9	12.5	3
14	12.4	13.6	3
21	9.4	12.4	3
22	8.3	11.5	4
23	8.9	10.7	4
$\bar{X} \pm \text{SEM}$	10.0 $\pm$ 0.6	12.1 $\pm$ 0.4	

Table 3 Plasma insulin, plasma-C-peptide, blood glucose and plasma- β -hydroxybutyrate ($\bar{X} \pm \text{SEM}$) in the "high" and "low" insulin binding groups. For definition see Results.

	"High binders" n=7	"Low binders" n=17
Insulin (mU/L)	8 $\pm$ 2	7 $\pm$ 2
C-peptide (nmol/L)	0.18 $\pm$ 0.04	0.16 $\pm$ 0.03
Blood glucose (mmol/L)	20.4 $\pm$ 3.4	20.8 $\pm$ 3.4
β -hydroxybutyrate (nmol/L)	1.749 $\pm$ 0.680	3.625 $\pm$ 1.108

In the individuals studied twice ($n=8$), the insulin binding increased from a pre-treatment value of 10.0% (range 8.3–13.2) to 12.1% (range 10.7–13.6) ($P < 0.01$), following 3–6 weeks of therapy (Table 2).

Plasma insulin and plasma C-peptide concentrations varied markedly among the diabetics, ranging from undetectable to 31 mU/L for insulin, and from undetectable to 0.39 nmol/L for C-peptide. No correlation was found between insulin concentrations and insulin binding to erythrocytes. Neither was any correlation found between insulin binding and plasma C-peptide, or blood glucose, or plasma β -hydroxybutyrate.

The diabetic group was subdivided into "high binders" and "low binders", according to whether the insulin binding was higher than or lower than the mean of the reference group (11.6%). No correlation was found between the insulin binding and insulin levels in either group. No difference was found between the groups with regard to plasma insulin, blood glucose, plasma C-peptide, or plasma β -hydroxybutyrate (Table 3).

The mean insulin binding did not differ between patients with plasma β -hydroxybutyrate of more than 1 mmol/L and less than 1 mmol/L ($\bar{X} = 10.0 \pm 0.5$ and 10.4 ± 0.7 , respectively). No difference in insulin binding was found in patients with plasma insulin of < 3 mU/L, compared with those with plasma insulin ≥ 8 mU/L ($\bar{X} = 9.8 \pm 0.5$ and 10.3 ± 0.7 respectively). When comparing the patients with the highest (> 20 mmol/L) and the lowest (< 10 mmol/L) blood glucose concentrations, there was no significant difference in insulin binding ($\bar{X} = 10.4 \pm 0.6$ and 10.3 ± 0.9 , respectively).

The reticulocyte count in the diabetics was $17 \pm 2 \times 10^9/L$, compared with $8 \pm 1 \times 10^9/L$ in the normal group ($P < 0.01$). Neither within the diabetics, nor within the normals, was any correlation found between insulin binding and the reticulocyte count. In the group of diabetics tested twice, the mean of the reticulocyte count was initially $21 \pm 3 \times 10^9/L$, and $13 \pm 1 \times 10^9/L$ on the later occasion ($P < 0.05$).

Discussion

Before institution of insulin therapy, insulin binding to erythrocytes was lower in the children with type I diabetes than in normal children. The variability of the binding was somewhat larger in the diabetic group. After a few weeks of insulin therapy, binding had increased to a normal level. The affinity of the binding did not differ between diabetics and normals, which implies a decreased number of receptors in the erythrocytes of diabetics during this phase of the disease.

These results are somewhat different from those reported by Pedersen et al. (1982) who studied a group of mainly adult diabetics. Their essential finding was a considerably wider variation of the binding in diabetics than in normals, but with a normal mean value. Because of the wide variation they descriptively classified the diabetics into two subpopulations, "high" and "low" binders, the "high binders" having higher plasma ketones and lower plasma insulin

values than the "low binders". Comparing the degree of metabolic disturbance in our patients on admission to hospital and in those of Pedersen et al. (1982), clearly that of our patients was less severe; in this respect, our patients correspond to their "low binders". The normalization of the low insulin binding following therapy in our study is in agreement with the findings of Pedersen et al. (1982) in their "low" binding group. Kappy et al. have previously shown insulin binding in treated diabetic children to be normal (Kappy and Plotnick 1979).

It has been shown that immature erythrocytes contain more insulin receptors than mature red cells (Eng, Lee and Yalow 1980; Ivarsson and Thorell 1981). The occurrence of a higher reticulocyte count in the diabetic group excludes the possibility of a lower proportion of immature red cells being the cause of the low binding in diabetics.

According to current theories on insulin receptor regulation, the low insulin levels in the diabetics would have been expected to induce an increased receptor concentration. However, not all hypoinsulinaemic states cause increased insulin binding. In growth hormone deficiency, insulin receptors remain unchanged, despite the reduction in circulating insulin (Lippe, Kaplan, Golden, Hendricks and Scott 1981). The reason for the lacking up-regulation of the insulin receptors in the untreated diabetics is uncertain. No studies have been reported on the mechanisms of up-regulation but it may be assumed that the low receptor concentration is a result of metabolic disorder in the peripheral cells, invalidating the mechanisms which would normally cause the up-regulation. The normalization of binding following improvement in the diabetic condition after therapy supports this theory. An alternative mechanism for the low receptor content is suggested in a recent report showing that in a group of juvenile onset diabetics 10 out of 22 had circulating auto-antibodies to the insulin receptor (Maron et al. 1983). The purified antibody competed effectively with the insulin binding to adipocytes. If the presence of such antibodies is a common phenomenon in juvenile diabetics one may expect that they would compete with any type of insulin receptor and therefore cause a decreased binding of ^{125}I -insulin in any competitive binding assay. To what an extent such a mechanism also would affect the function of the receptor *in vivo* is still unknown. The increase of the receptor concentration following insulin treatment found in our cases might thus be an effect of diminishing concentration of these antibodies.

We have previously demonstrated that insulin resistance occurs in the initial phase of diabetes if combined with a severe metabolic disturbance, and that insulin sensitivity becomes normalized after several weeks of insulin therapy*. The increase of the receptor concentration following therapy found in the present study, suggests a role of the receptors in this connection.

* Ivarsson et al.: Unpublished results.

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Erythrocyte insulin receptors and insulin sensitivity in adrenocortical insufficiency. Report of a case of diabetes mellitus type I with superimposed Addison's disease

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Abstract. Studies on erythrocyte insulin receptors were performed in a boy with type I diabetes mellitus and superimposed adrenocortical insufficiency before and during treatment with hydrocortisone and 9- α -fluorohydrocortisone. The development of the adrenal insufficiency was associated with a progressively increased sensitivity to insulin which reverted after therapy. The maximum specific insulin binding of the erythrocytes was low during threatening addisonian crisis (6.9%) but normalized during hydrocortisone treatment (12.0%). These findings suggest that the increased insulin sensitivity characteristic for adrenocortical insufficiency is not an effect of an increased insulin receptor concentration and that hypocortisolaemia is associated with a down-regulation of the insulin receptors.

A striking clinical feature of patients with diabetes mellitus and superimposed Addison's disease is their increased sensitivity to insulin, which in turn leads to a decreased insulin requirement and even life-threatening hypoglycaemic episodes. The mechanism of the insulin hypersensitivity is poorly understood and there are no reports in man of the effect of a chronically low corticosteroid plasma level on insulin receptors. Studies on fat cells from adrenalectomized rats have shown conflicting results with either increased (Häring et al. 1980) or unchanged binding (Olefsky et al. 1975). On the other hand recent studies have shown in vitro (Goldfine et al. 1973; Kahn et al. 1978) and in vivo (Kahn et al. 1978; Olefsky et al. 1975) that glucocorticoid in excess depresses insulin binding, suggesting that the insulin receptors play a role in steroid-induced insulin resistance.

We had the opportunity of following a patient with insulin dependent diabetes mellitus during the development of adrenal insufficiency and of measuring the binding of insulin to erythrocytes before and after substitution with hydrocortisone and 9- α -fluorohydrocortisone.

Subject and Methods

Patient

The patient was an eighteen-year-old boy who developed type I diabetes mellitus at the age of 14. At that time rather widespread vitiligo had also been noticed. There was no family history of diabetes mellitus, though his mother had necrobiosis lipoidica but without any evidence of diabetes. He had been satisfactorily treated with a diabetic diet and insulin, the last 2 years in a dose of 40 U Monotard® a day.

During the last 4 months before admission he had been anorectic, developed weakness and steadily lost weight. He had had several hypoglycaemic attacks despite successive reduction of the insulin dosage. In retrospect he admitted a slight increase in his desire for salt, but no orthostatic symptoms. One month before admission he had been treated at another hospital because of a severe hypoglycaemic attack with convulsions followed by mental confusion lasting several days.

On admission to the hospital his general condition was fairly satisfactory. There was no increased pigmentation of the skin or the oral mucosa but widespread vitiligo. Blood pressure was 110/80 mmHg. Serum electrolytes: Na 140, K 5.6, Ca 2.30, standard CO₂ 24 mmol/l. The

serum creatinine was 88 $\mu\text{mol/l}$. The plasma levels of T_3 , T_4 and TSH were normal. Basal serum cortisol 70, 60, 50 and 40 nmol/l at 07, 13, 19 and 01 h (normal range 200–800, 100–800, 50–600 and 20–400, respectively). Plasma ACTH was 120 pmol/l (normal 30 ± 26), plasma aldosterone 0.02 nmol/l (normal 0.14–0.85) and plasma renin 9.75 $\mu\text{g/l} \cdot 3 \text{ h}$ (normal 2.1–4.0). Basal 24 h urinary cortisol excretion was undetectable (less than 10 nmol/24 h).

Oral metyrapone induced no increase in 24 h urinary 17-hydroxy-corticoid excretion, but caused an incipient addisonian crisis with hypotension, vomiting and somnolence. Treatment with hydrocortisone (15 mg twice a day) and 9- α -fluoro-hydrocortisone (0.05 mg a day) was initiated. An ACTH stimulation test (60 U Acton® by iv infusion for 8 h) had no demonstrable effect on the very low urine excretion of cortisol.

No serum autoantibodies were demonstrated against adrenal or thyroid tissues, gastric parietal cells or smooth muscle. Serum immunoglobulins were normal. HLA-typing showed A9, 25; B18; Bw 35; Cw 4. A tuberculin skin test was negative. Roentgenography of the chest and abdomen revealed no abnormal calcifications.

Corticoid substitution therapy was attended by an increase in the insulin requirement from 28 U/day to 56 U/day. The patient left hospital in good general condition and the course has since been uneventful (12 months' observation period).

Methods

Insulin binding to erythrocytes was measured as described by Gambhir et al. (1977) with a few modifications (Ivarsson & Thorell 1981). The specific cell bound fraction was adjusted to a standard erythrocyte concentration of 4.4×10^{12} cells/l. Binding data were calculated from plots of the specific bound radioactivity versus bound/free (B/F) ratio according to Scatchard (1949). The reticulocytes were counted as described by Atwaker & Erslev (1972).

Results

The maximum specific insulin binding to erythrocytes measured 2 days before the incipient addisonian crisis was 6.9% (normal 11.6 ± 1.6 SD). The reticulocyte count was $36 \times 10^9/\text{l}$. During treatment with hydrocortisone (30 mg/day) the maximum specific binding was 12.0% (Fig. 1). The reticulocyte count was $43 \times 10^9/\text{l}$. K_e calculated according De Meyts & Roth (1975) was $5.5 \times 10^8 \text{ M}^{-1}$ before and $7.0 \times 10^8 \text{ M}^{-1}$ after treatment. The corresponding values for K_f were $0.7 \times 10^8 \text{ M}^{-1}$ and $1.0 \times 10^8 \text{ M}^{-1}$ respectively. The binding capacity was $0.62 \times 10^{-10} \text{ mol/l}$ and increased to $1.25 \times 10^{-10} \text{ mol/l}$.

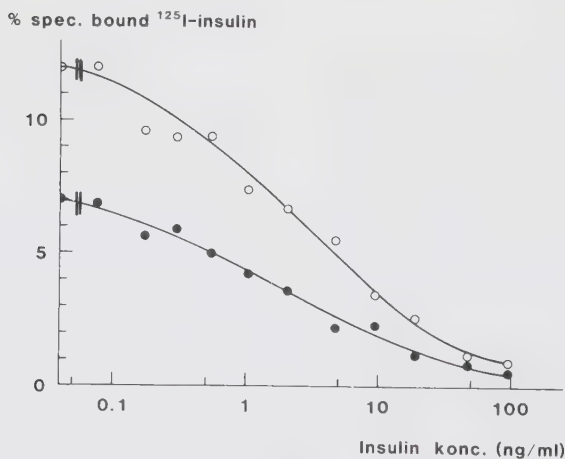


Fig. 1.

The per cent of [^{125}I]insulin bound by RBC at various insulin concentrations before ● and during ○ corticoid treatment.

Discussion

There is much clinical and experimental evidence that adrenocortical insufficiency or adrenalectomy is followed by increased insulin sensitivity whereas hypercorticism is associated with a state of insulin resistance. The mechanisms of these events are still obscure. No direct measurement of insulin receptors in adrenocortical insufficiency has been reported. Some studies suggest that the insulin receptors might play a role for the insulin resistance in hypercorticism though the results have been conflicting. The administration of prednisone to normal individuals induced a marked increase in the number of insulin receptors on monocytes (Beck-Nielsen et al. 1980) whereas other studies of human erythrocytes after dexamethasone and prednisone ingestion (Yasuda & Kitabchi 1980) and of human monocytes after administration of dexamethasone and cortisone (De Pirro et al. 1980) showed a reduced insulin binding. In a recent study (Fantus et al. 1981) prednisone administration had no effect on insulin binding to lymphocytes though the incubation of lymphocytes with hydrocortisone *in vitro* caused an increase in binding.

Our patient who developed primary adrenal insufficiency superimposed on type I diabetes mellitus exhibited a low insulin binding to erythrocytes during a period with low insulin requirement. Following substitution therapy with hydrocortisone and 9- α -fluoro-hydrocortisone the insulin dosage had to be increased markedly. During this treatment the insulin receptors increased to normal concentration. According to current theories of insulin receptor function one would have expected the insulin binding to be high (up-regulation) during a period of low insulin levels and to decrease as a consequence of increasing hyperinsulinaemia (down-regulation) (Gavin et al. 1974; Olefsky & Reaven 1977). However, the presence of many other uncontrolled variables in insulin treated diabetes complicates the interpretation of the change in the insulin binding. In ketoacidotic diabetics both low and high insulin binding has been described (Pedersen et al. 1982). Insulin treatment increased the receptor levels in the diabetics with low receptors while the effect was the opposite in the high receptor group. Furthermore though the insulin receptors are elevated in some hypoinsulinaemic conditions such as anorexia nervosa (Wachslight-Rodbard et al. 1979) and pancreatic

insufficiency (Nosadini et al. 1982), they are unchanged when the low insulin levels are combined with growth hormone deficiency (Lippe et al. 1981).

Another uncertain variable is whether it is possible to extrapolate results of receptor studies on erythrocytes to other cell types in the body. It has, however, been found that erythrocyte insulin receptors exhibited the same general insulin binding characteristics as their counterparts in liver, adipocytes and monocytes (Gambhir et al. 1978; Kappy & Plotnick 1979) and they mirror changes in receptor concentration and affinity found in adipocytes (Kolterman et al. 1979) and monocytes (Wachslight-Rodbard et al. 1979) in certain clinical conditions. In addition the erythrocyte insulin binding has been found to vary with the age of the cells (Eng et al. 1980; Ivarsson & Thorell 1981) but the reticulocyte counts were unchanged in all sample for insulin receptor in this case.

Despite the obvious difficulties to draw firm conclusions about the corticoid effects on the receptor levels we interpret the paradoxical results that the increased insulin sensitivity of adrenal insufficiency is probably not an effect of the alterations at the receptor level. Similarly the lack of consistency of the results in the receptor studies during hypercorticism support the view that glucocorticoid induced insulin resistance is not induced at the receptor. This is supported by a recent study by Rizza et al. (1982), which proposed that the decreased insulin sensitivity of hypercortisolaemia is due to a post-receptor defect. If the increased insulin sensitivity of hypocortisolaemia also is a post-receptor effect, a down-regulation of the receptors may be regarded as a physiological adjustment which reduces the insulin effect in a state of decreased availability of glucose.

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INSULIN SENSITIVITY IN THE INITIAL PHASE OF TYPE I
DIABETES MELLITUS

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SUMMARY The blood glucose response to a brief infusion of insulin (0.012 U/kg body weight) was studied in the initial phase of type I diabetes in 21 children and in 20 healthy controls. The insulin effect was impaired in most of the diabetics and the mean blood glucose decrement was $14.3 \pm 3.5\%$ (SEM) as compared with $20.9 \pm 2.1\%$ in the normals. Subgrouping the diabetics according to the severity of the diabetic condition revealed normal (or even supranormal) sensitivity in the diabetics with mild metabolic derangement (mean blood glucose decrement $36.2 \pm 6.0\%$). In children with more disturbed metabolism mean blood glucose decrement was only $5.6 \pm 0.8\%$ indicating a marked insulin resistance. Further evidence for a relationship between the insulin sensitivity and the severity of the diabetic state was found in the correlations between the percentual blood glucose decrement and the fasting blood glucose ($r = -0.79$, $p < 0.01$), the glucose assimilation rate ($r = 0.71$, $p < 0.01$), as well as the blood glucose level at 120 minutes during the OGTT ($r = -0.76$, $p < 0.01$). Four of the insulin resistant patients were re-tested during remission and exhibited then normal insulin sensitivity.

KEY WORDS: Insulin sensitivity, diabetes mellitus type I

While insulin resistance is a recognized feature of type II diabetes (for references see 1,2), the findings of studies on insulin sensitivity in type I diabetes have been less consistent, some demonstrating peripheral resistance (3,4,5,6,7), others normal sensitivity (5,6,8,9). Such divergent results may have been due to differences in methodology and to heterogeneity in the patient groups studied.

Several studies have shown that a decline in the first-phase insulin response to glucose stimulation is one of the earliest aberrations found during the progress into clinically overt type I diabetes mellitus (10,11). Alterations in the effect of insulin on glucose utilization at the onset of type I diabetes may therefore primarily be found in the responsiveness to acute perturbations in the insulin levels rather than in influence during steady state conditions. We have therefore studied the insulin sensitivity with an experimental model which measures the change in blood glucose after a brief infusion of insulin. This induces a hyperinsulinaemia at physiological plasma insulin levels and reproducibly measures the insulin effect in a dose-related fashion (12,9).

One of the major differences between patient groups is related to the severity of the diabetic state. Therefore, we studied the relation between insulin effect and variables reflecting the degree of metabolic disturbance, such as hyperglycaemia, ketonuria and glucose assimilation rate during intravenous and oral glucose tolerance tests.

SUBJECTS AND METHODS

Patients. Twenty-one juvenile diabetics (7 girls and 14 boys) were studied. Relevant clinical data are given in Table 1. All the patients required continued insulin therapy. None were acidotic, i.e. with a standard CO_2 less than 20 mmol/L. Ten had ketonuria, i.e. more than 1+ with Ketostix[®].

The insulin effect was tested at the onset of their diabetes mellitus within the first week of admission to hospital in 17 cases, and within the second week in the remaining four.

Table 1

Clinical data and glucose tolerance tests

Patient number	Sex	Age yrs	Weight kg	Fasting blood glucose at admission mmol/L	IVGTT		OGTT			
					k_G	% / min	60 minutes		120 minutes	
							Blood glucose mmol/L	Insulin mU/L	Blood glucose mmol/L	Insulin mU/L
1	M	5	16	10.7	-	-	14.1	11	7.8	13
2	M	13	50	28.0	0.7	0.7	-	-	-	-
3	M	8	28	15.7	0.8	0.8	14.5	21	13.9	21
4	M	14	52	25.1	0.6	0.6	12.5	29	15.6	21
5	F	9	26	10.2	0.9	0.9	-	-	-	-
6	M	10	32	11.7	0.9	0.9	12.7	10	13.2	8
7	F	15	70	17.6	0.6	0.6	17.1	23	17.1	23
8	F	9	24	15.1	0.8	0.8	-	-	-	-
9	F	13	35	16.4	0.7	0.7	23.4	12	22.6	15
10	M	11	42	18.9	0.4	0.4	26.1	3	22.7	<3
11	M	13	47	20.0	0.3	0.3	-	-	-	-
12	F	13	44	43.8	0.5	0.5	24.0	10	26.8	15
13	M	14	39	33.0	0.6	0.6	18.4	14	18.6	19
14	F	13	46	19.9	0.6	0.6	21.5	10	21.9	10
15	M	15	39	21.5	0.3	0.3	21.5	14	18.7	10
16	M	14	55	41.0	0.4	0.4	18.8	7	19.1	4

17	F	8	20	54.5	0.6	18.3	6	17.6	6
18	M	13	35	27.1	0.5	24.1	7	23.0	8
19	M	15	44	42.9	0.3	25.7	3	24.8	5
20	M	12	32	25.5	0.5	15.5	15	14.6	8
21	M	11	35	29.5	0.4	23.1	6	20.7	9

Mean +SEM Control	11.8±0.6	38.6±2.8	0.57±0.04*	19.5±1.1 ^o	12±2	18.7±1.2**	12±2
Mean +SEM	12.0±0.2	44.4±2.1	2.11±0.14				

* Mean value for 1-6 and 7-21 were 0.78±0.06 and 0.50±0.04 respectively (p<0.01)
^o Mean value for 1-6 and 7-21 were 13.5±0.5 and 21.3±0.9 respectively (p<0.01)
 ** Mean value for 1-6 and 7-21 were 12.6±1.7 and 20.6±1.0 respectively (p<0.01)

Five of the patients were re-tested 3 to 11 months later, when they were in a remission phase (13).

Controls. Twenty healthy children volunteered (8 girls and 12 boys). They were 10-14 years of age, within ± 2 SD of ideal weight (14) and with no first degree relative with known diabetes. They were instructed not to change their diet, but to be sure to eat at least 250 g carbohydrates per day for the last seven days before the test.

Verbal and written informed consent was obtained from both the children and their parents. The project was approved by the Medical Ethics Committee, University of Lund.

Study protocol. The tests were performed in the morning after an overnight fast with minimal physical exercise, in the case of the diabetics 24 hours after their last dose of an intermediate-acting insulin preparation. An indwelling catheter was inserted into an antecubital vein, and clotting prevented by a slow infusion of 0.9% sodium chloride. After starting the infusion there was a pre-test period of at least 30 minutes to ensure a steady state. No anticoagulant was injected, nor was any present in the syringes used for sampling, but EDTA with natrium fluoride was added to the test tubes as an anticoagulant. Plasma for the insulin, glucagon, and human growth hormone assays was stored at -20°C until analysed. The tests were performed on three consecutive days.

Insulin infusion. Transient hyperinsulinaemia was induced by infusion of 0.012 U/kg body weight of insulin (Actrapid[®], Novo, Copenhagen, Denmark) at a steady rate for 2 minutes, the infusion and sampling of blood being carefully timed from the start. Blood was drawn for measuring glucose and insulin at -10, -5 and -1 minutes and at 5, 10, 20, 30, 45 and 60 minutes after the injection. The insulin effect was calculated as the decrement of blood glucose from the mean of the three pre-test values to the 20 minute value (Δ -glucose).

In three diabetics a control study was made in which the insulin was replaced by the same volume of 0.9% sodium chloride.

Glucose tolerance tests. The intravenous glucose tolerance test (IVGTT) was performed with 0.5 g glucose per kg body weight. The glucose assimilation rate was estimated from the graph of the log blood glucose versus time, and expressed as percentual fall per minute (k_G). The oral glucose tolerance test (OGTT) was performed only in the diabetics. The glucose dose was 30 g per m^2 body surface in a 10% solution to be taken within 3 minutes. For statistical analysis the blood glucose values at 60 minutes (OGTT₆₀) and 120 minutes (OGTT₁₂₀) were used.

Methods. Blood glucose was measured in triplicate by a glucose-oxidase method (15) and by a hexokinase method (16). As the linear regression between the glucose-oxidase method (G_G) and the hexokinase method (G_H) was: $G_G = G_H \times 1.20 - 0.70$, the hexokinase data were corrected accordingly. Insulin was measured in plasma by a radioimmunoassay (17). All patients were tested for insulin antibodies but the result was invariably negative. Glucagon was measured according to Faloona and Unger (18) and human growth hormone according to Thorell and Larson (19).

Statistical methods. Results for groups were expressed as mean $\pm$ standard error of mean. Student's t-test, paired Student's t-test and linear regression analysis were used for statistical analysis.

RESULTS

The infusion of insulin raised plasma insulin from a basal level of 8 ± 1 mU/L to a peak value of 62 ± 3 mU/L in the diabetic group. In the control group the basal insulin level was 14 ± 2 mU/L and the peak value 76 ± 5 mU/L. The difference in the increase of plasma insulin between the two groups was not significant ($p > 0.10$).

In the healthy children the hyperinsulinaemia caused a fall in blood glucose from 4.6 ± 0.1 mmol/L to a nadir of 3.6 ± 0.1 mmol/L at 20 minutes, corresponding to a decrease of $20.9 \pm 2.1\%$ (range 10-40%) (Table 2). After 20 minutes the glucose concentration

gradually returned to its pre-test level, and at 60 minutes it was 4.4 ± 0.1 mmol/L.

In the diabetic group the hyperinsulinemia caused a slow decrease in blood glucose from 11.3 ± 1.0 mmol/L to 10.2 ± 1.1 mmol/L at 20 minutes, after which it remained essentially constant. The relative decrement was $14.3 \pm 3.5\%$ (range 0-53%) (Table 2). In neither group was there any relationship between the increase in the insulin value and Δ -glucose. The infusion of saline did not affect the blood glucose level (15.6 mmol/L at 0 minutes and 15.5 mmol/L at 20 minutes).

The effect of the insulin infusion varied, depending on the severity of the diabetic condition. There were significant correlations between the percentual blood glucose decrement and the degree of fasting hyperglycemia ($r = -0.79$, $p < 0.01$) (Fig. 1), as well as to the k_G -IVGTT ($r = 0.71$, $p < 0.01$) and OGTT₁₂₀ ($r = -0.76$, $p < 0.01$), respectively. Using the absolute blood glucose decrement the significances remained.

Analysis of the individual glucose responses showed distinct differences between patients with fasting blood glucose either higher or lower than 8.0 mmol/L. In addition, these subgroups differed significantly in their values for k_G -IVGTT, OGTT₆₀ and OGTT₁₂₀ (Table 1).

In the group with a less severe diabetic condition, the blood glucose response to insulin infusion showed the same pattern as in the healthy children with a distinct nadir at 20 minutes. The blood glucose decrement was 2.0 ± 0.4 mmol/L or $36.2 \pm 6.0\%$ (range 18-53%), which was more pronounced than in the control group ($p < 0.05$). In the group with a more severe diabetic state the insulin effect was markedly reduced, the response being delayed and the curve continuing to decline slowly for 60 minutes. In this group the blood glucose decrement was only 0.7 ± 0.1 mmol/L or $5.6 \pm 0.8\%$ (range 0-11%), which is markedly less than that in the mild diabetics ($p < 0.01$). The difference in the increase of plasma insulin between these subgroups was not significant, nor was there any correlation between the peak insulin value during the infusion and the Δ -glucose. The occurrence of ketonuria did not significantly influence the blood glucose response.

Table 2

Insulin infusion test

No	Blood glucose [*] mmol/L	Ketonuria [*]	<u>Glucose decrement at 20 min.</u>	
			mmol/L	%
1	4.0	0	2.0	50
2	4.5	0	1.4	31
3	4.6	0	2.0	43
4	6.1	++	1.1	18
5	6.5	++	1.4	22
6	7.2	0	3.8	53
7	8.5	0	0.6	7
8	9.1	0	1.0	11
9	10.6	+++	0.7	7
10	11.5	+++	0.6	5
11	13.4	0	1.1	8
12	13.6	+++	0	0
13	13.8	0	1.1	8
14	14.1	+++	1.1	8
15	14.2	++	0.3	2
16	14.9	0	0	0
17	15.3	+	0.6	4
18	15.4	+++	1.0	6
19	15.6	++	0.6	4
20	16.7	+++	1.2	7
21	17.4	+	1.3	7

Mean

± SEM 11.3±1.0

1.1±0.2

14.3±3.5

Control

Mean

± SEM 4.6±0.1

1.0±0.1

20.9±2.1

* In the morning of the insulin infusion test.

In none of the groups did the plasma levels of growth hormone or glucagon show any changes during the insulin infusion test indicating no hormonal counter regulation during the time period studied (Table 3).

Of five patients re-tested during the remission period, four who had been markedly hyperglycaemic and insulin resistant at the initial test now showed a normal glucose response to the insulin infusion. The blood glucose decrement was 2.0 ± 0.1 mmol/L or 23% (range 19-36%), at this stage compared with 0.7 ± 0.2 mmol/L, or 6% (range 2-8%), in the initial phase ($p < 0.01$). One child with a fasting blood glucose of 4.5 mmol/L at the initial test showed a normal response on both test occasions (31% and 25%, respectively).

DISCUSSION

This study demonstrates that in the initial phase of type I diabetes, the insulin effect varies with the severity of the diabetic state. In the diabetic children with minimal metabolic disturbance the pattern of the glucose response to the insulin infusion was the same as that of normal children. The mean decrement of blood glucose was even higher than that found in the control group. In the diabetics with more disturbed metabolism and evidence of pronounced insulin deficiency the insulin effect was both retarded and reduced, indicating a resistance to insulin. The relationship between the insulin effect and the severity of the diabetic condition was further substantiated by the correlations between the blood glucose decrement and the fasting blood glucose concentration, the glucose assimilation rate and the blood glucose levels at 60 and 120 minutes during the OGTT.

In earlier studies type I diabetes was shown to be associated either with insulin resistance (3,4,7), or with normal insulin sensitivity (8,9), or to constitute a mixed population in this respect (5,6). Reassessing these previous studies by relating the insulin effect to the severity of the diabetic state of the patient using the blood glucose level as an indicator supports our results. In the studies showing marked insulin resistance, the

Table 3

Plasma levels of growth hormone and glucagon during insulin infusion test (Mean $\pm$ SEM)

		-5	10	60 minutes
hGH (μ g/L)	Patients	6 $\pm$ 1	6 $\pm$ 2	7 $\pm$ 1
	Controls	3 $\pm$ 1	3 $\pm$ 1	4 $\pm$ 1
Glucagon (pmol/L)	Patients	47 $\pm$ 6	45 $\pm$ 6	43 $\pm$ 6
	Controls	36 $\pm$ 4	41 $\pm$ 4	38 $\pm$ 4

mean blood glucose concentrations were high (20.0 mmol/L (3), 16.2 mmol/L (4) and 10.0 mmol/L (7)). Studies by Martin and Stocks (5) indicate a tendency to insulin resistance in subjects with high fasting blood glucose but normal sensitivity at lower fasting blood glucose concentration. Others have found normal insulin sensitivity in diabetics with low fasting blood glucose (8,9) and in the group of 12 patients described by Ginsberg (6) nine had normal insulin sensitivity, the three resistant patients being the only ones who had fasting blood glucose concentrations above 10 mmol/L.

On the other hand, an increased concentration of blood glucose per se is not associated with insulin resistance as shown with the technique used in this study (12) and in several other studies with different techniques (20,21,22,23).

The return of a normal response to insulin after a period of insulin therapy with improvement of metabolic control, also indicates that insulin resistance is not an abnormality associated with type I diabetes mellitus per se. This is in agreement with previous studies on severely ketoacidotic diabetics (24).

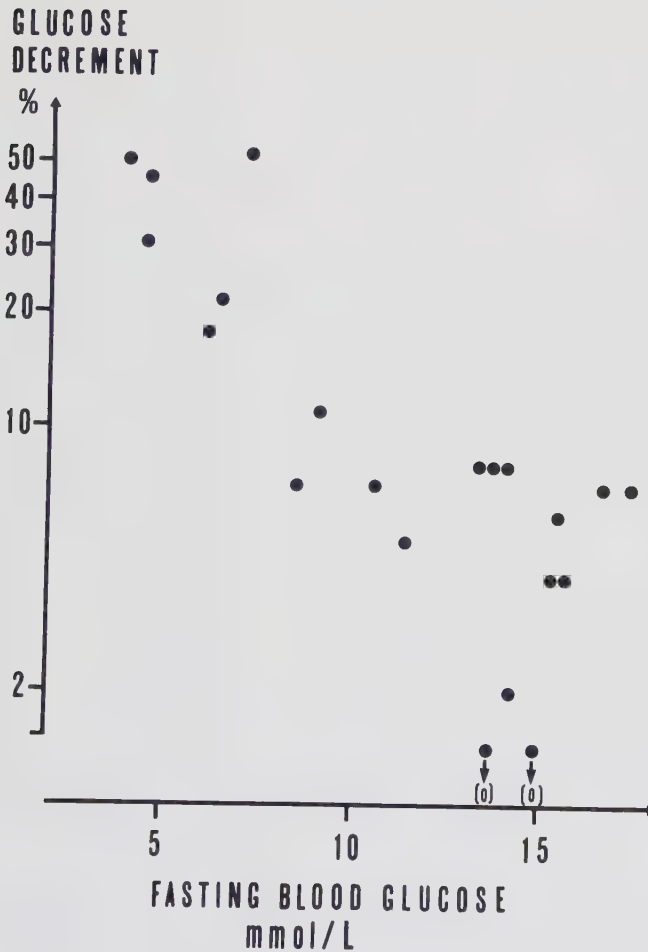
Insulin resistance may be caused by a variety of mechanisms. A decreased insulin receptor concentration has been suggested as the cause of insulin resistance in obesity (25), uremia (26), and acromegaly (27). In a separate study we have found no connection between the metabolic state and the insulin binding to erythrocytes at the onset of type I diabetes (28). Randle et al. (29) suggested that high concentrations of fatty acids and ketone bodies in plasma or intracellularly may interfere with glucose metabolism and cause insulin resistance. The occurrence of marked insulin resistance, also in cases without ketonuria, indicates that insulin resistance may develop even in the absence of marked ketoacidosis.

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Legend to figure

Fig 1. Glucose decrement (%) following insulin infusion versus fasting blood glucose in the diabetic children ($r=-0.79$, $p<0.01$).



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